Universidade de Aveiro Departamento de Química

Ano 2013

RITA PINHEIRO LOPES

EFEITO DA ALTA PRESSÃO NA PRODUÇÃO DE IOGURTE

EFFECTS OF HIGH HYDROSTATIC PRESSURE ON YOGURT PRODUCTION

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Ivonne Delgadillo Giraldo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho ao meu pai, minha mãe e ao meu namorado pelo constante apoio e ajuda.

o júri

presidente

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palavras-chave Alta pressão, fermentação, bactérias ácido-lácticas, iogurte.

resumo

Este trabalho teve como objetivo o estudo da aplicação da tecnologia de Alta Pressão ao processo de produção do iogurte.

Para isso, a fermentação foi realizada sob diferentes condições de pressão, utilizando iogurte natural como inóculo. A monitorização deste processo foi realizada recorrendo à análise de diversos parâmetros físico-químicos (acidez titulável, pH, concentração de açúcares redutores e outros mais específicos, como concentração de D-glucose, ácidos L- e D-láctico, acetaldeído e etanol). Também foi realizada uma análise microbiológica a *Streptococcus thermophilus* e *Lactobacillus bulgaricus* (bactérias fermentativas do inóculo utilizado) de modo a inferir a sua viabilidade durante as fermentações testadas. Pela análise físico-química, conclui-se que o aumento da pressão influencia negativamente a velocidade fermentativa, sendo que sob 100 MPa o processo fermentativo é completamente inibido. Foi também realizada uma análise cinética, onde foi verificado que a acidez titulável era o parâmetro menos afetado pelo aumento da pressão.

Adicionalmente, foram realizadas fermentações à pressão atmosférica com pré-tratamentos variáveis de pressão (50 MPa ou 100 MPa durante 90 ou 180 minutos). Em todos os pré-tratamentos testados não houve fermentação, mas depois, a fermentação começa à pressão atmosférica, sendo que a sua velocidade depende das condições do pré-tratamento utilizado (pressão/tempo). No pré-tratamento de 100 MPa durante 90 minutos houve um aumento considerável da velocidade fermentativa, sendo mais rápida que a fermentação sem pré-tratamento, enquanto que com um pré-tratamento mais longo, a velocidade fermentativa diminui.

Pela análise da concentração de D-glucose, verifica-se que quando não há fermentação, há uma maior concentração de D-glucose no meio, o que pode ser explicado pela hidrólise da lactose presente no leite. A concentração dos dois isómeros de ácido láctico está de acordo com os resultados obtidos para a acidez titulável (aumenta com o tempo de fermentação), e adicionalmente verifica-se que no iogurte, o isómero L- está em maior quantidade do que o isómero D-. O acetaldeído está presente numa baixa concentração no iogurte e a sua produção também é inibida com o aumento da pressão, tal como acontece com outros produtos da fermentação. Quanto ao etanol, não foi possível quantificar pelo método utilizado.

Com a análise microbiológica realizada verificou-se que o aumento da pressão inibe o crescimento das bactérias fermentativas. Para além disso, verificou-se que *S. thermophilus* é mais resistente à pressão do que *L. bulgaricus* e está presente em maiores quantidades no iogurte.

keywords High pressure, fermentation, lactic acid bacteria, yogurt.

abstract

The purpose of this work was the study of high pressure technology application on set yogurt's production process.

For that, the fermentation process was performed under different pressure conditions, using set yogurt as inoculum. In order to monitor product formation and substrate consumption over the fermentation time, analyses were performed for several physicochemical parameters (titratable acidity, pH variation, reducing sugars concentration and parameters more specific as D-glucose, L- and D-lactic acids, acetaldehyde and ethanol concentrations). A microbiological analysis to *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (starter cultures of set yogurt) was also performed in order to evaluate its viability during fermentation under the pressure conditions tested.

By analyzing general physicochemical parameters, it was possible to conclude that increasing fermentation pressure influences negatively the fermentation rate and with pressures around 100 MPa, the fermentative process was totally inhibited. Through a kinetic analysis, it was verified that titratable acidity was the parameter lesser affected by increasing fermentation pressure.

In addition, fermentation at atmospheric pressure with variable pressure pre-treatments (50 or 100 MPa for 90 or 180 minutes) was performed. In all pre-treatments tested in this work, there was no fermentation during pre-treatment, but at atmospheric pressure the fermentation occurs and its rate depends of pre-treatment conditions (pressure and time). With a pre-treatment of 100 MPa for 90 minutes there has a significant increase of fermentative rate, became faster than fermentation without pre-treatment, but when the pre-treatment time increases, the fermentation rate also increases.

With D-glucose concentration analysis, it was verified that when fermentation stops, D-glucose concentration increases, which can be explained by milk's lactose hydrolysis. Lactic acid isomers concentration are in accordance to titratable acidity results obtained (increasing over fermentation time) and it was verified that L-lactic acid is present in higher amount than D- isomer. Acetaldehyde was present in small amounts in yogurt and its production was inhibited with the increasing pressure, as the others fermentation products analyzed. In what regards to ethanol production, it was not possible to quantify by the analytical method applied.

The microbiological analysis indicated that increasing pressure inhibits starters' growth. *S. thermophilus* was more resistant to pressure than *L. bulgaricus* and the former one was present in a higher amount in yogurt.

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I. INTRODUCTION

1. YOGURT

1.1. Contextualization

Despite of the desirable sensory characteristics and nutritional value of fermented dairy products, they were originally developed to preserve milk, since during fermentation there is lactic acid production and acidification. Currently, the term fermented dairy product is used to indicate the products that are prepared using lactic acid bacteria (LAB) starter cultures and a controlled fermentation. LAB utilize the nutrients in milk to support their growth and subsequent fermentation where lactic acid is produced, which reduces the pH of these products and inhibits the growth of many pathogenic and spoilage microorganisms. Taking into account that there are several starter cultures that can be used in fermentation, there is a great variety of fermented dairy products with a diversity of flavor and textural attributes, including cheese, yogurt, buttermilk, butter, acidophilus milk, and sour cream [1, 2].

Yogurt is defined as a coagulated milk product that results from the fermentation of lactose in milk by 2 thermophilic lactic acid bacteria that live together symbiotically, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, where a mixture of LAB species is carefully selected to complement each other and to achieve a remarkable efficiency in acid production [3-5]. To meet the USA Yogurt Association's criteria for "live and active culture yogurt," the finished yogurt product must contain live LAB in amounts $\geq 10^8$ organisms/g at the time of manufacture, and the cultures must remain active at the end of the stated shelf life [6, 7].

The formation of lactic acid in yogurt's production lowers the pH of milk which cause coagulation of the casein micelles into a three-dimensional network structure. As the pH decreases to less than 5.3, colloidal calcium phosphate is solubilized from the casein micelle, causing dissociation of micelles, aggregation of casein proteins and its precipitation at the casein's isoelectric point (pH 4.6) and whey is trapped [1, 8, 9]. The resulting product has a gel-like texture and characteristic tang (due production of lactic acid from lactose) [2, 8, 10].

1.2.Historical Relevance of Yogurt

The origin of cultured dairy products dates back to the dawn of civilization; they are mentioned in the Bible and the sacred books of Hinduism. Many of products mentioned are still widely consumed and they had often been used therapeutically before the existence of bacteria was recognized [11].

At the beginning of the 20th century the main functions of gut flora were completely unknown. In 1907, Ilya Ilyich Metchnikoff, the Nobel prize winner in Medicine in 1908, postulated that the bacteria involved in yogurt production, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, suppress the putrefactive-type fermentations of the intestinal flora and consumption of these yogurts played a role in maintaining health [12]. Therefore, the first mass production of yogurt was started by the pharmacist Isaac Carasso, with the goal of combating intestinal infections [13].

Yogurt and similar fermented milk products have been very popular for a long time in Mediterranean countries, central and southwest Asia and central Europe, and yogurt is still manufactured using traditional procedures in many of these countries. Since the last world war, yogurt consumption has been steadily increasing not only in European countries, but also in the United States, enhancing its industrial-scale production. At present, new types of fermented milk are available, prepared by adding fruits or flavouring, enriched with vitamins or containing selected intestinal bacteria such as *Lactobacillus acidophilus* and several *Bifidobacterium* species [6, 14, 15].

During the last few years, the contribution of biotechnology to yogurt's production has been very important as it offers the possibility of selecting and using new microorganisms' sources to increase the yield of sources already used, to introduce specific functional properties in raw materials or ingredients, to improve the nutritional value and the bioavailability of nutrients and flavor [13].

Nowadays, in many modern societies, fermented dairy products make up a substantial proportion of the total daily food consumption. Due to yogurt high nutritional value and health benefits, this fermented dairy product has gained special prominence and economic importance, which was reflected in yogurt's consumption that steadily increased over the last 30 years in the U.S.A. (Economic Research Service 2002) and in other parts of the world [5, 16]. So, according to Harry Balzer (vice-president of the market research

firm The NPD Group), yogurt earning the title of food of the decade due to its increasing popularity [17].

1.3.Yogurt's Lactic Acid Bacteria: Characteristics and Metabolism

Regarding to yogurt starter cultures, they are composed by two thermophilic lactic acid bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus.*

S. salivarius subsp *thermophilus* is the equivalent name proposed to designate *Streptococcus thermophilus* which was originally described by Orla-Jensen (1919) [18]. It is exclusively isolated from the dairy environment, ferments only few carbohydrates, i.e. lactose, sucrose, glucose and sometimes galactose, and is characterized by its thermoresistance and a rather high growth temperature which may reach 50-52 ^{o}C [19].

L. delbrueckii subsp *bulgaricus* was also first described by Orla-Jensen (1919) [18]. *Lactobacillus bulgaricus* is homofermentative, ferments few carbohydrates, i.e. glucose, lactose, fructose, and sometimes galactose or mannose, and has a high growth temperature (up to 48 or 50 ^{o}C) [20].

Concerning to starter shape, *Lactobacillus bulgaricus* are rod with rounded ends and *Streptococcus thermophilus* has a spherical to ovoid shape with an irregular segments (Figure 1) [19]**.** Both are Gram-positive, facultative anaerobic, non-motile and non-spore-forming bacteria [20]**.**

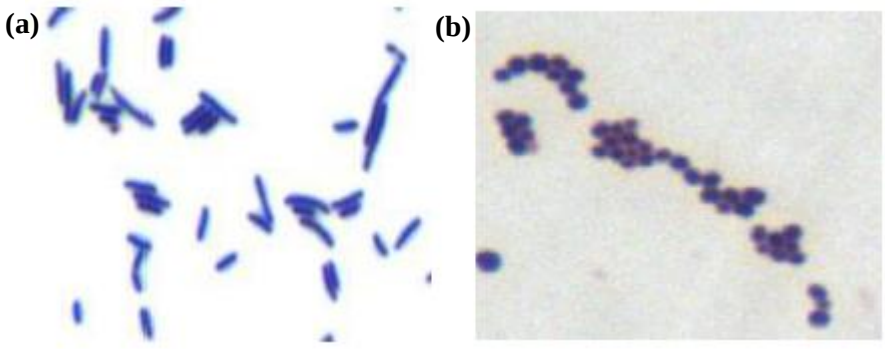

Figure 1. Microscopic images of *Lactobacillus bulgaricus* (a) and *Streptococcus thermophilus* (b). (Adapted from [21])

The role of streptococci and lactobacilli in yogurt manufacture can be summarized as milk acidification, synthesis of aromatic compounds, development of texture and viscosity. Thus, for industrial yogurt manufacture, starter selection takes into account these 3 properties [6].

1.3.1.1.LAB's Symbiotic Relations

In a mixed culture, it is observed a positive interaction between *S. thermophilus* and *L. bulgaricus*, which leads to stimulation of each other growth and acid production, that is much larger than the sum of single cultures [22, 23]. Perez *et al.* (1991) [24] suggested that the symbiotic relationship between S. *thermophilus* and *L. bulgaricus* is very important for yogurt and cheese production because it decreases fermentation time.

In addition, total proteolysis in mixed culture exceeds the sum of the values obtained by each strain alone [25]. Mixed yogurt cultures may also stimulate the production of some metabolites such as acetaldehyde [26, 27] and influence carbohydrate utilization. For instance, one *L. bulgaricus* strain studied which cannot use galactose in pure culture metabolizes this sugar when it is associated with one strain of *S. thermophilus* [28, 29]. In other hand, *L. bulgaricus* has an important protease activity and hydrolyzes the milk proteins to small peptides and amino acids. These peptides and amino acids enhance the growth of *S. thermophilus*, which has limited proteolytic activity. Furthermore, *S. thermophilus* metabolizes pyruvic acid to formic acid and carbon dioxide, which in turn, stimulates the growth of *L. bulgaricus.* So, initially, *S. thermophilus* grows faster than *L. bulgaricus*; however, at the later stages of the fermentation process, the growth of the *S. thermophilus* is inhibited by the reduced pH of the yogurt. The mutual stimulation of the yogurt cultures through their metabolic activity significantly increases the formation of lactic acid to a rate greater than would be possible by the individual cultures [1].

1.3.1.2.Metabolism of Carbohydrate

The main metabolic pathways of lactic acid bacteria involved in yogurt production are represented in Figure 2.

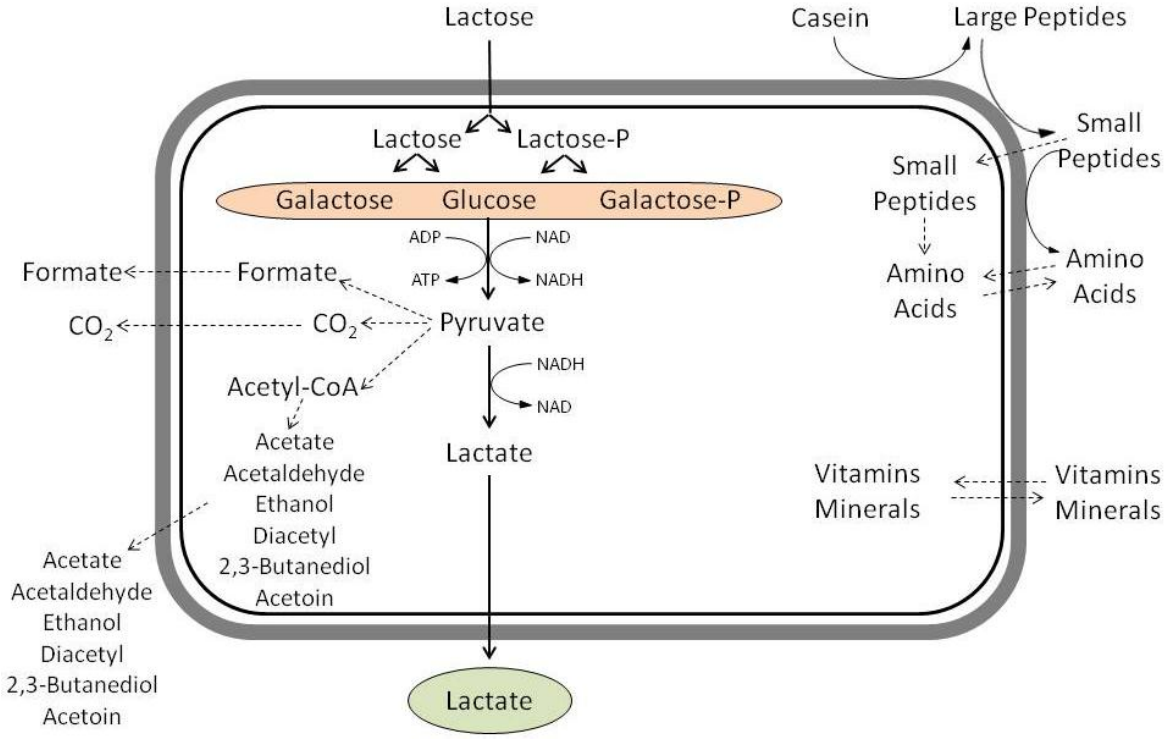

Figure 2. Metabolic pathways of lactic acid bacteria (Adapted from [30]).

Regarding to carbohydrate's metabolism, the LAB possesses two different systems for lactose transport into the cell, that are necessary to fermentation: a phosphotranspherase system (PTS) and a permease system, both requiring energy [31]. In the PTS, the energy is derived from phosphoenolpyruvate (PEP) and this system is also known as PEP:PTS, while the required energy for lactose transport in the permease system is derived from adenosine triphosphate (ATP). Lactose is transported in the form of lactose phosphate (lactose-P) in the PTS as a result of the transfer of a high-energy phosphate from PEP to lactose, while in the permease system, it is transported without any transformation. The thermophilic starter bacteria such as streptococci and lactobacilli use a permease system for lactose transport, but some may also contain PTS [32, 33]. When lactose is inside the cell, the enzymes involved in its initial metabolism are phospho-β-galactosidase (P-β-gal) and β- galactosidase (β-gal) for lactose transported by PTS and permease system, respectively [34].

S. thermophilus only possess β-gal that hydrolyses lactose to glucose and galactose that are subsequently fermented via the Embden–Meyerhof–Parnas (glycolytic) and Leloir pathways, respectively. In glycolytic pathway, *S. thermophilus* possesses 2 fructose-1,6-

diphosphate-independent (FDP-independent) lactate dehydrogenases (LDH) which reduce pyruvate to lactic acid. In the Leloir pathway, galactose is transformed to glucose-1-P that is converted to glucose-6-P that enters in the glycolytic pathway [34].

L. bulgaricus also possess the PTS system and, therefore, have P-β-gal that hydrolyses lactose-P to glucose and galactose-6-P, which are subsequently fermented via the Embden–Meyerhof–Parnas (glycolytic) and tagatose pathways, respectively. In the tagatose pathway, galactose-6-P is metabolized through several derivatives of tagatose (a stereoisomer of fructose) to glyceraldehydes-3-P and dihydroxyacetone-P [6, 34]. But this lactobacillus uses rather the glucose moiety of lactose than galactose moiety that releases into the growth medium. However some strains can use galactose in a growth medium when there are limiting concentrations of lactose [6].

Products of both the tagatose and Leloir pathways are further metabolised through the glycolytic pathway, with lactic acid as the end-product (Figure 2). Key steps in the formation of lactic acid from the monosaccharides include the hydrolysis of the hexose diphosphates to glyceraldehyde-3-phosphate by aldolases, the formation of pyruvate from phosphoenol pyruvate by pyruvate kinase, and the reduction of pyruvate to lactic acid by lactate dehydrogenase [1, 34].

Regarding to sucrose's metabolism, this has been poorly documented, especially at the molecular and genetic levels. It is known that during the growth of *S. thermophilus* on sucrose, both glucose and fructose are used. However, fructose accumulates in the growth medium, even when the strain can use it [35]. Some studies reported an inhibitory effect of high sucrose content in milk (10-12%) on the growth of yogurt bacteria, which is due to both an adverse osmotic effect of the solutes in milk and a low water activity [36].

As stated previously, the main product of carbohydrate's metabolism is lactic acid. In yogurt's production, both lactic acid isomers are simultaneously produced, because *S. thermophilus* possesses 2 FDP-independent L-LDH [37, 38] and produces mainly L(+)-lactic acid and *L. bulgaricus* produces mainly D(-)-lactic acid since possesses an NAD-dependent stereospecific LDH [39]. D(-)-lactic acid is metabolized very slowly in man, compared to the L(+)-isomer, therefore may cause metabolic disorders if ingested in excess. Thus, is must be used industrial starters with a lower proportion of *L. bulgaricus* in production of yogurt [6].

1.3.1.3.Production of Polysaccharides

In addition to their primary role (production of lactic acid to lower pH), certain strains of lactic acid bacteria make a further contribution to the physical structure of yogurt [40, 41] by production of extracellular polysaccharides (EPS). In stirred yogurt, yogurt beverages and low milk solids yogurts, production of polysaccharides can improve viscosity and texture, increase resistance to mechanical handling and decrease susceptibility to syneresis (the expelling of interstitial liquid due to association of the protein molecules and shrinkage of a gel network on yogurt) [6, 8].

Lactic acid bacteria are therefore classified as 'ropy' or 'non-ropy' depending on whether or not they express EPS. The quantities of polymer formed by ropy strains of both species vary considerably even under identical experimental conditions [42-44]. It is difficult to establish a good correlation between the quantity of polysaccharide produced and the corresponding viscosity, which may be due to changes in the 3-dimensional configuration of polymers and to their interactions with some milk compounds, mainly caseins that are precipitated at low pH [45].

1.3.1.4.Enzymatic Activities

1.3.1.4.1. Proteolytic Activity

In yogurt, proteolysis is not determinative for organoleptic properties. On the other hand, proteolytic activity is greatly involved in both nutrition and interactions of yogurt bacteria, since lactic acid bacteria cannot synthesize essential amino acids. Therefore, they need an extracellular nitrogen source, which are mainly milk proteins as caseins and whey proteins (through action of extracellular proteinases, membrane bound aminopeptidases and intracellular exopeptidases and proteinases) and the low molecular weight peptide fraction of milk (through action of enzymes with leucine-aminopeptidase and arginine-amino-peptidase activity) [6].

1.3.1.4.2. Lipolytic Activity

Lipolysis is generally low in yogurt and is therefore not significant in terms of flavor. The free fatty acid content of yogurt differs only slightly from that of milk [46, 47].

1.3.1.4.3. Urease Activity

In milk under some conditions, *S. thermophilus* produces a large amount of carbon dioxide (CO_2) which is not formed from lactose metabolism since this microorganism is a strictly homofermentative species [22]. In addition, it is also produced a large quantity of ammonia (NH_3), which can be explained by activity of urease, which breaks down milk urea (about 250 $mg.L^{-1}$) into CO_2 and NH_3 [48-50]. This leads to the alkalinization of the growth medium and directly affects acidification rate measurements in milk [44, 51, 52].

Therefore, urease activity has technological interest for two reasons: (i) it enables streptococcal count in mixed cultures with *L. bulgaricus*, by measuring the amount of CO_2 produced, since this lactobacillus has no urease activity [49, 53] and (ii) NH_3 production affects the evaluation of streptococcal acidifying properties by pH measurements [6].

1.3.1.5.Production of Antimicrobial Compounds by Yogurt's Bacteria

As mentioned above, there is generally a symbiotic relationship between yogurt bacteria, but growth inhibition is sometimes observed [54-56]. This inhibition may be due to competition for one or more nutrients of the growth medium [57] or to inhibitory compounds produced by the strains, such as bacteriocins and inhibitory peptides [55]. Thus, this growth inhibition should be taken into account when the yogurt starters are selected.

1.4.Yogurt's Composition

During yogurt production, there is an extensive formation of compounds that constitute the biochemical and nutritional composition of yogurt.

The nutrient composition of yogurt is based on the nutrient composition of the milk from which it is derived, its processing conditions and its storage. In addition, the nutritional and physiologic value of the finished yogurt product is also affected by changes in milk constituents during lactic acid fermentation, the species and strains of bacteria used, the source and type of milk solids that may be added to mixture and the temperature and duration of fermentation process [5].

The characteristic flavor of yogurt is due to lactic acid and various carbonyl compounds, i.e. acetaldehyde, acetone, diacetyl, ethanal, dimethylsulfide and acetic acid, produced by *S. thermophilus* and *L. bulgaricus* (Figure 2). Other carbonyl substances have

also been detected, like 1-octen-3-one and 1-nonen-3-one which are considered important odorants. In addition to carbonyl substances, others volatile compounds have also been identified in yogurt, i.e. volatile fatty acids [58, 59] and several compounds derived from the thermal degradation of lipids, lactose and proteins during the heat treatment of milk before yogurt manufacture [60].

Acetaldehyde is considered as the major flavor component of yogurt [58, 61, 62] and diacetyl contributes to the delicate, full flavor of yogurt and seems to be important when the acetaldehyde content is low [63]. Acetaldehyde formation may occur during the Embden-Meyerhof-Parnas pathway, that utilize glucose and generates pyruvate, and its formation is catalyzed by a α-carboxylase. Other way to acetaldehyde formation is the catalysis by an aldehyde dehydrogenase of acetyl-CoA, which is formed from pyruvate by the action of a pyruvate dehydrogenase. Threonine aldolase can also produce this compound because catalyzes the cleavage of threonine to acetaldehyde and glycine and this way appears to be the most important pathway for acetaldehyde production in yogurt. In addition, acetaldehyde can be formed from DNA components because there are strains of *S. thermophilus* and *L. bulgaricus* (studied by Raya et. al (1986) [64]) that possess a deoxyriboaldolase which catalyzes the synthesis of acetaldehyde from 2-deoxyribose-5-phosphate. On other hand, diacetyl is formed from citrate, which should occur spontaneously or be catalyzed by an α-acetolactate oxidase [3, 6].

In addition, dairy products like yogurt are an exclusive source of the disaccharide lactose or its monosaccharides (glucose and galactose) in human diets. Before fermentation, the lactose content of the yogurt mix is generally ≈ 6% [7], but during the fermentative process occurs hydrolysis of 20–30% of lactose to its absorbable monosaccharide components, glucose and galactose [4], and a portion of the glucose is converted to lactic acid. Therefore, usually, this hydrolysis results in lower lactose concentrations in yogurt than in milk, which in part explains why yogurt is tolerated better than milk by persons with lactose maldigestion [65-67].

Regarding to protein content, yogurt has a high level of free amino acids, especially proline and glycine [68]. In addition, yogurt contains whey proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA) and immunoglobulin) which remain soluble at low pH values [5, 8, 69].

Other yogurt constituents are lipids, but since it has a low lipase activity, free fatty acids are released in very small amounts [7]. However, conjugated linoleic acid (CLA), a long-chain biohydrogenated derivative of linoleic acid, is present in yogurt [70], which was hypothesized that results from the biohydrogenation that occurs during fermentation [71].

In addition, yogurt is an excellent source of minerals, as calcium and phosphorus [72].

1.5.Yogurt's Health Benefits

The nutritional value of a particular food depends on its digestibility and its content of essential nutrients, which may be improved by fermentation due to enzymatic activity of the microbial culture. Yogurt has been known for its nutritional value, nutraceutical, therapeutic, and probiotic effects, such as digestion enhancement, immune system boosting, anticarcinogenic activity, and reduction of serum cholesterol [13, 73].

As mentioned above, fermentation can improve nutrient digestibility, so may greatly improve the tolerance for certain foods. Yogurt has a lower lactose content than milk thus, yogurt is better tolerated than milk by lactose-deficient individuals, which is considered a yogurt benefit [5, 13, 74-77].

During yogurt production, there are others essential nutrients that may be synthesized, such as vitamins, amino acids, proteins and others biologically active compounds (Figure 2), which includes bacteria used for fermentation, their metabolic products and components derived from milk [3, 13].

In addition, lactic acid bacteria have a strong inhibitory effect against the growth and toxin production of most other bacteria, namely spoilage organisms and pathogens that can contaminate food. This inhibitory activity can be the result of competition for the available nutrients, decrease in redox potential, decrease in pH resulting from production of lactic acid and acetic acid, production of other inhibitory primary metabolites (as hydrogen peroxide, carbon dioxide or diacetyl) and production of special antimicrobial compounds (as bacteriocins and antibiotics) [13].

Furthermore, research revealed that certain strains of *S. thermophilus* and *L. bulgaricus* are capable of assimilating considerable amount of cholesterol and reduces total cholesterol, high-density lipoprotein (HDLP) cholesterol and low-density lipoprotein (LDLP) cholesterol [74, 78, 79].

1.6.Yogurt's Industrial Production

The steps of industrial production of yogurt are presented schematically in Figure 3.

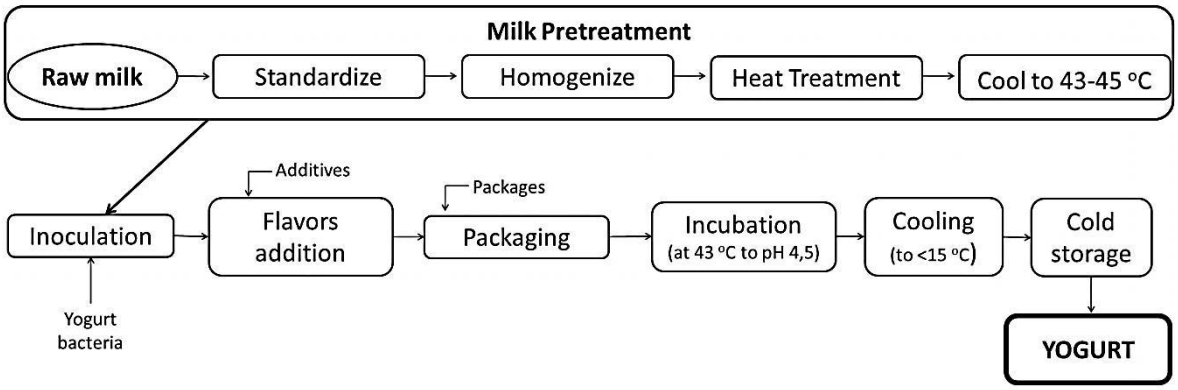

Figure 3. Processing scheme for yogurt. (Adapted from [1])

In an initial phase, milk used for yogurt production is subjected to several pretreatment operations in order to create growth conditions for bacteria culture and improve the aspect and consistency of yogurt [80]. First, milk is standardized to the desired fat (0.5–3.5% fat) and milk solids-not-fat (12.5%) content. Then, it is performed a milk homogenization that consists in decrease and homogenize the size of the fat globule. This step also changes the milk proteins, increasing firmness and reducing syneresis - undesirable in yogurt [81]. In a final phase of milk pre-treatment, it is proceeded to a heat treatment in order to eliminate pathogenic microorganisms, reducing also the oxygen content of milk to provide a good growth medium for the starter cultures [81].

Following heat treatment, the milk is cooled to 43–45°C for inoculation of the starter cultures and the subsequent incubation is conducted by addition of 1.5–3% of the operating culture (mixed culture of *S. thermophilus* and *L. bulgaricus*) at 42–45 ◦C for about 3 h [1, 3]. But, incubation time varies according to process temperature, which can be short (40–45°C for 2–3 hours) or long (30°C for 16–18 hours). In the end, the final product should has a pH value of about 4.5 and contains 0.7–1.1% of lactic acid [1, 3]. When these conditions are reached, the final product is cooled and stored (at 5°C) in order to slowdown the physical, chemical and microbiological degradation and stop fermentation [82].

1.6.1. Yogurt's Types: Definition and Industrial Production

Figure 4 shows the industrial process of several types of yogurt. As we can see, for all types of yogurt, the milk pre-treatment is equal, only changing the production process after starter inoculation.

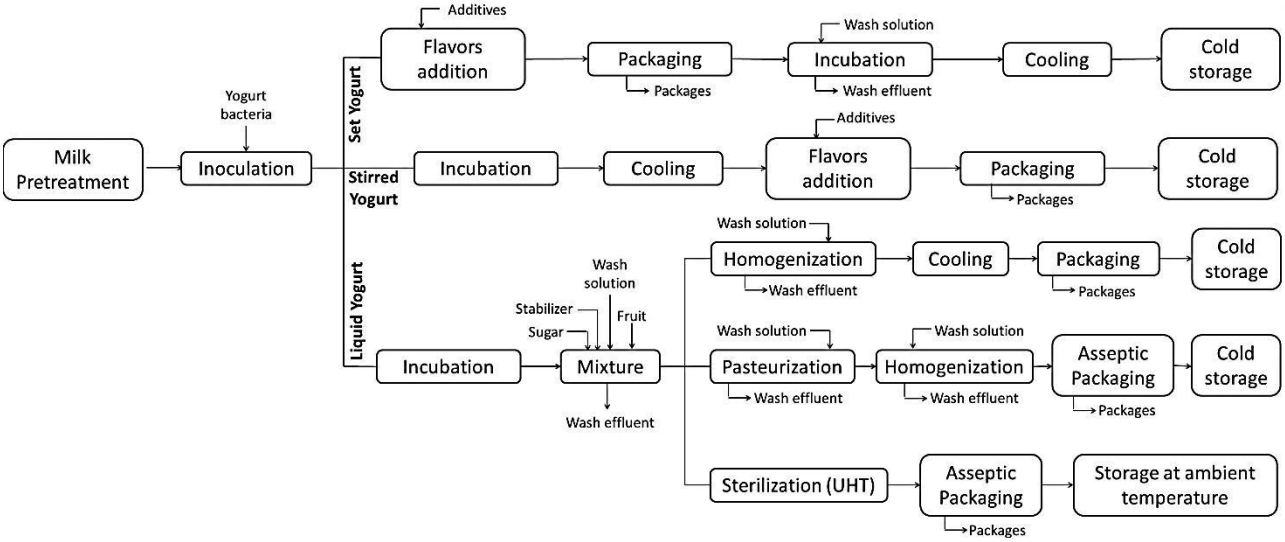

Figure 4. Industrial process diagram for several types of yogurt. (Adapted from [80])

The types of yogurt can be classified as [80]:

- Stirred yogurt (incubation in tanks and cooling before packaging);
- Set yogurt (incubation and cooling in the package);
- Liquid yogurt (clot is liquid before packaging);
- Ice yogurt (incubation in tanks and frozen like ice cream);
- Concentrated yogurt (incubation in tanks, concentration and cooling before packaging).

The most popular types are set, stirred and liquid yogurt, so their production processes are described below.

1.6.1.1.Stirred Yogurt

In stirred yogurt's production process, the incubation with starter cultures is performed in isothermic tanks of maturation, with pH-meter to control mixture pH, during

2.5-3 hours at 42-43 ^{o}C. During tank filling and incubation, the mixture is stirred, to ensure an uniform acid gel [80]. When the mixture reaches the ideal pH, the final product is cooled until 15-22 ^{o}C in order to stop fermentation. During the latter phase, the clot must be subject to a mild stirring to improve the yogurt body [80].

The additives and fruit pulp addition to yogurt is done after the yogurt cooling, which is performed continuously by pumping the obtained yogurt and additives through a static mixer. Before the additives and fruit pulp addition, they have to be pasteurized in order to eliminate microorganisms, but it is important that this process does not change the taste and texture of fruit. In this yogurt's type, the packaging of yogurt on a proper packages is only performed at the end of process [8, 80].

1.6.1.2.Set Yogurt

The production operations of stirred and set yogurts are similar, only changing their order (Figure 4) [80]. Additives and fruit pulp are added continuously to milk flow before packaging and incubation with starter cultures. The latter operation takes place after yogurt packaging in an incubation chamber for fermentation and after the mix reaches the ideal pH, the packages are cooled until the desired final temperature (<5 ^{o}C). For this yogurt type is important not stirring the packages during incubation time (3-3.5 hours), so that the final product be a gel that forms a firm and unbroken coagulum [1, 80].

1.6.1.3.Liquid Yogurt

To obtain this type of yogurt, clot is broken before being cooled and bottled, inducing considerable changes on rheological properties [83]. The addition of stabilizers, fruit and sugar is performed after the mixture and cooling to 18-20 ^{o}C. To increase yogurt's shelf-life, the mix undergoes to different thermic treatments and aseptic packaging, as it can be verified in Figure 4 [80].

1.6.1.4.Others Types

Probiotic yogurts are yogurts that the only difference in their production process is the bacteria cultures added, since in addition to yogurt starter cultures also contain probiotic cultures, such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. These

bacteria are claimed to enhance the growth of beneficial bacteria in the intestine and thus have beneficial effects to consumer, namely probiotic effects [10].

Strained (Greek style) yogurt is other yogurt type that is not represented in Figure 4 and have been became popular recently. This yogurt type is prepared by removing some of the whey by straining through a cloth or by centrifugation [10].

2. HIGH HYDROSTATIC PRESSURE (HHP)

2.1 HHP Definition

High Hydrostatic Pressure (HHP) is one "new" or emerging technology receiving a great deal of attention lately [84].

Hydrostatic pressure is a key physical parameter in the biosphere that ranges from 0.1 MPa (atmospheric pressure) at sea level to more than 110 MPa in ocean depths. So, pressure is considered a variable of life which has influenced the evolution and distribution of both microorganisms and macroorganisms, since different magnitudes of pressure exert different effects on organisms. Thus the ability to adapt to pressure changes of one kind or another is a characteristic of all life. [85-87].

During the past decades, an increasing number of scientific disciplines have started to explore the potential of exposing biological systems of varying complexity to HHP. And the unique effects that pressure exerts on biological systems are currently being investigated at different levels, ranging from proteins, enzymes and viruses to microorganisms, mammalian cells and tissues. So, the knowledge and understanding of high pressure effects on these increasingly complex systems is steadily growing, which leads to detection of several unique HHP applications in bioscience over the past few years [85, 86].

2.2.Principles of HHP Technology

The pressurization is carried out during the pressure treatment in a confined space (pressure vessel) containing a fluid (usually water), which acts as the pressure transmitting medium and is utilized to facilitate the operation and compatibility with materials [88, 89].

The HHP technology is in accordance with the two operating principles described below:

- Le Chatelier's principle: Any chemical reaction that is accompanied by decrease in volume can be enhanced by pressure and vice-versa [90].
- Isostatic principle: The transmittance of pressure is uniform and instantaneous (independent of size and geometry of product) [90].

As stated by Le Chatelier, HHP affects any reaction, conformational change, or phase transition that is accompanied by a decrease in volume, which will be favored at high pressures, while reactions involving an increase in volume will be inhibited [91, 92]. However, owing to the complexity of foods and the possibility of changes and reactions that can occur under pressure, predictions of the effects of HHP treatments are difficult, as are generalizations about any particular type of food. However, a tremendous amount of information has been generated in the past decade, as evidenced by the effects of HHP on food systems, including microbial inactivation, chemical and enzymatic reactions, and structure and functionality of biopolymers [92-94].

The second principle stated that high-pressure treatments are independent of product size and geometry, and their effect is uniform and instantaneous, as shown in Figure 5 [95-98]. According to this principle, the product is compressed by uniform pressure from every direction and then returns to its original shape when the pressure is released [99]. This is one of the advantages offered by this processing technology because thermal processing are dependent of geometry and size of product and frequently lead to size reduction to improve the processing [88].

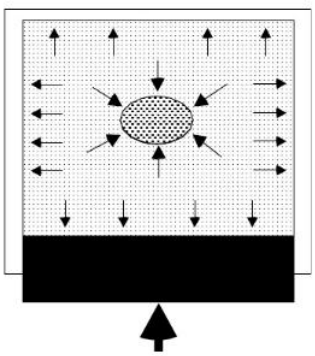

Figure 5. The principal of isostatic processing [84].

The pressure is held for the desired treatment time and then released. The applied pressure and the holding time will depend on the type of product treated and the expected final result [88].

2.3.Pressure Effects on Microorganisms

HHP challenges life because it forces a decrease in volume, so several cellular components suffer structural modifications favoring a more compact form. Besides the structural alterations in biomolecules, pressure also disturbs the equilibrium of chemical reactions towards volume reduction [87]. Therefore HHP can have several effects in microbial cells and its components, which some examples are represented in Figure 6.

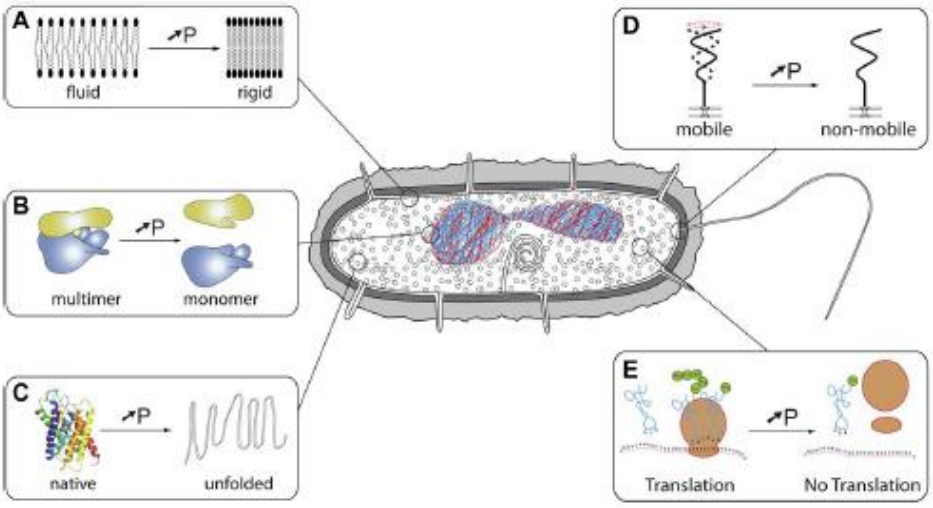

Figure 6. Examples of the effects of high hydrostatic pressure on cells and cellular components. A: lipids in membranes; B: multimeric protein assemblages. C: protein structure; D: cellular motility; E: protein translation by ribosomes [100].

As represented in Figure 6, the lipid bilayers became rigid with increasing pressure due to loss of membrane fluidity. Thus, the lipid membranes became rapidly impermeable to water and other molecules, and protein-lipid interactions essential to the optimal function of the membrane are weakened [101]. However, more recently other competing theories have been advanced for the functional significance of membrane fatty acid modulation with temperature including the maintenance of ion permeability for

bioenergetic purposes [102] and the adjustment of membrane curvature elastic stress [103], and these could be also applied to high pressure stress [104].

In addition, it is known that changes in temperature, hydrostatic pressure and solute composition disrupted non-covalent "weak" chemical bonds, which are essential to maintain protein structure and function. Therefore, the increasing of pressure is sufficient to affect protein multimer association and stability, as well as catalytic sites, therefore protein functions are altered with compression [105-108]. Furthermore, a few investigations have dealt with protein expression after HHP treatment [109-111] and verified that exposure cells to pressure caused an increase in the expression level of pressure induced proteins [112].

Subunit dissociation of bacterial cells ribosomes (Figure 6) seems to be one of the major factors of the cell death by HHP, since bacterial cells only survive until the number of functional ribosomes decreases below a threshold level [113]. In addition, high pressures also cause changes in DNA structure and function, namely its stabilization by pressure increasing. The double- to single-strand transition necessary for cell processes, like replication, transcription and translation, may become more difficult with pressure increase because the transition temperature increases [114].

Therefore, when submitted to increasing hydrostatic pressure, organisms experience the failure of several of their cellular functions (Figure 6), like loss of flagellar motility; loss of protein and nucleic acid synthesis; loss of enzymatic function and metabolism; and alterations in cellular architecture, etc. which eventually leads to cell death [100].

In addition, bacterial cells incubated at elevated pressures become filamentous, which could result from pressure effects on DNA replication or condensation as described above. Cell division is also indirectly influenced by pressure, since the activity of several division proteins should be possible targets of HHP [86, 115-119].

Regarding to damage magnitude of HHP application, this is dependent of several factors, as varying degree of organisms' tolerance, the pressure extent and duration, and it can lead to cell death. For example, microbial growth is inhibited when it is applied pressures between 20 and 130 MPa, and pressures above 130 MPa usually results in microbial cell death. But, surprisingly, some living organisms are able to withstand such high-pressure environments and survive despite the strong effect of this stress on cell structures and environments their functions [109, 120].

2.4.HHP Applications

2.4.1. Conventional HHP Applications

HHP technology was originally used in the production of ceramics, steels, and super alloys. But, in the past years, high-pressure technology is of increasing interest for use in biological and food systems [73, 84].

The first reported use of high pressures as a method of food processing was in 1899 at the West Virginia University in the USA [121], where experiments were conducted using HHP to preserve milk, fruit juice, meat and a variety of fruits. They demonstrated that microorganisms in these products could be destroyed by pressures of 658 MPa (6500 atm) for 10 minutes [122]. Later in 1990, Meidi-ya Food Co. (Osaka, Japan) introduced to the market apple, strawberry, and kiwi jams, which were pasteurized using only HHP technology [123, 124]. Some of the foods currently processed by HHP and already commercialized are jams, fruit juice (tomato juice) [125], meat, oysters, ham, fruit jellies and pourable salad dressings, salsa and poultry [126]. Some of these products are represented in Figure 7.

Figure 7. Commercial high-pressure processed products marketed in Japan, Europe and the United States [127].

This processing technology emerge due to an consumer demand for minimally processed, additive-free, shelf-stable products, and it was used as an alternative to traditional treatments such as freezing, canning or drying that rely on heating or cooling operations. These technologies may contribute to the degradation of various food quality attributes (as color, flavor and texture), in spite of ensure a high level of food safety [128].

In addition to the advantages of HHP in food processing, this technology can be used to process both liquid and solid (water-containing) foods and besides destroy microorganisms, HHP adds others benefits to the foods [129] such as (i) extends shelf-life, (ii) ponders additive free and fresh food, (ii) manipulates the texture and (iv) enhances desired attributes (digestibility) [130].

2.4.2. Novel HHP Applications

Currently, the widest application of HHP processes within the food industry is still extending the shelf-life of food products, but other uses for this technology have been described. These include solute diffusion processes (salting, sugaring), assisted freezing-thawing processes, modification of functional properties of proteins and other macromolecules and compounds extraction of cells because pressure increase compounds permeability and solubility [88, 131, 132].

While inactivation of microorganisms by lethal HHP is well investigated in the context of food preservation and the hygienic safety of minimal food processes, sub-lethal HHP stress response and its effect on adaptation and cross-protection is less understood and it can bring numerous interesting applications in biotechnology. One of these applications is the realization of fermentative processes/microbial growth under pressure [85, 86], But there are still few studies about the effect of this application of HHP in fermentative processes. For example, Picard *et al*. (2007) [133] monitor alcoholic fermentation *in situ* in order to study the energetic metabolism of the yeast *S. cerevisiae* under HHP (up to 100 MPa). At 10 MPa, fermentation proceeds three times faster than at ambient pressure and the fermentation yield is enhanced by 5% after 24 h. These results show that there was an increase of yield and rate of alcoholic fermentation by *S. cerevisiae*, when the fermentation occurs under sub-lethal pressures. Besides the rate increase of fermentation under pressure, the bacteria metabolism can be also changed when HHP is applied. Bothun *et al.*, (2004) [134] studied the effect of HHP on *Clostridium*

thermocellum cultures, a bacterium capable to produce ethanol from cellulosic material, but have low ethanol yield due to the formation of organics acids (i.e. acetate, lactate). In this study, the authors verified that cultures at 7.0 MPa and 17.3 MPa, the ethanol:acetate (E/A) ratios increase $>10^2$ relative to samples at atmospheric pressure and the cell growth was inhibited, thus there was a metabolism change during the fermentation under HHP. In addition to this HHP application, there is another and less documented reflection of bacterial adaptability to this stress, which corresponds to the microorganisms' capacity to develop resistance against it, while the wild type strain does not possess this characteristic. Mota *et. al.* [135] reviewed the studies performed that focus the both reflections for microbial growth under HHP.

2.5.Application of HHP on Yogurt

Regarding to the application of HHP on yogurt, two strategies have been used. The main purpose of both strategies is the quality improve of yogurt and its preservation by application of HHP, so there are studies of yogurt production from HHP-treated milk and yogurt pressurization to inactivate microorganisms, i. e. yogurt's pasteurization by HHP [136].

2.5.1. Milk HHP-Treatment

In milk pre-treatment, there is milk pasteurization, which can be based on HHP treatments of pressures between 300 and 600 MPa. These treatments cause inactivation of microorganisms including most infectious foodborne pathogens without causing many modifications of endogenous milk enzymes and important quality characteristics such as taste, flavor, color, vitamin and nutrient content [91, 137, 138].

For example, lactose in milk and milk products may isomerize to lactulose by heating and then degrade to form acids and other sugars. Lopez-Fandino *et al.*, (1996) [139] observed that there was no changes in these compounds after pressurization (100–400 MPa for 10–60 min at 25°C), suggesting no Maillard or lactose isomerization reaction occurs in milk after pressure treatment.

Harte *et al.* (2003) [140] reported that yogurt made from milk subjected to HHP (400–500 MPa) showed an increase on yield stress, elastic modulus and resistance to

normal penetration, while having syneresis reduction, compared with yogurts made from thermally treated milk (85 ^{o}C for 30 min) and from raw milk [141].

Later, Penna *et al.*, (2006) [16] studied the effect of milk processing on the microstructure of probiotic low-fat yogurt. For that, the authors carried out a study involving combined treatment of HHP (676 MPa for 5 min) and heat (85°C for 30 min) for low-fat yogurt, using different probiotic starter cultures. The authors reported that the obtained yogurt gel has a higher consistency index value along with acceptable rheological and textural properties. In 2007, Penna and his co-workers [73] made other study on low-fat yogurt prepared using similar HHP and thermal treatment conditions, which resulted in a dense aggregated protein structure with smooth surface, a compact gel with gel texture and viscosity improved, when compared to fewer interconnected chains in untreated yogurt.

According to Chicon *et al.*, (2008) [142], milk HHP treatment also enhances pepsin hydrolysis of β-lactoglobulin at 400 MPa. There is reduction in antigenicity and Immunoglobulin E (IgE) binding to β-lactoglobulin, which further opens the possibility of obtaining hypoallergenic hydrolysates of β-lactoglobulin.

Thus, the application of HHP in milk treatment offers a microbiologically safe and additive-free yogurt with improved characteristics, such as reduced syneresis, better texture, increased shelf life and high nutritional and sensory qualities [138, 140]. For instance, it was also reported that HHP improves acid coagulation of milk without detrimental effects on important quality characteristics [138].

2.5.2. Yogurt Pasteurization

As previously stated, HHP can be applied in yogurt production process in order to preserve the final product [143].

In 1999, Reps and his co-workers [144] found that yogurt's HHP treatment of 400 MPa completely inactivated *L. bulgaricus* while *S. thermophilus* was more resistant towards pressure. Later on 2008, Shah *et al.*[145] also studied the effect of HHP treatment on viable populations of *L. bulgaricus* and *S. thermophilus* and verified that at 480 MPa there was a reduction of the number of viable microorganisms and *L. bulgaricus* showed again the greatest sensitivity to HHP treatment. So, this study gives the idea that yogurt shelf-life can be enhanced by HHP treatment. Shah *et al.*, (2008) [145] also study the

viability recovery of organisms after one week of refrigerated storage and it was verified that there was a significant recovery in their viability.

In addition, Reps *et al.* (2009) [146] studied the possibility of applying the HHP technology for yogurt preservation and for that subjected yogurt to pressures of 200–700 MPa during 15 minutes at 18 ^{o}C. Pressurized yogurt was stored at temperatures of 4 and 20 ^{o}C for four weeks. As previously stated, the authors observed that application of high pressures considerably reduces the *L. bulgaricus* content, in this case it was used pressures exceeding 300 MPa and the bacteria content decreased to a level below the recommended by standards. In addition, pressurization caused a slight decrease in yogurt acidity, but during the storage period, the acidity of the pressurized yogurts remained constant, both at 4 and 20 ^{o}C. They also observed that application of pressures exceeding 200 MPa caused slight deterioration of yogurt consistency, whereas pressurization at more than 500 MPa significantly worsened the consistency. However, the color, taste and flavor of yogurts remained unchanged after HHP treatment and during the storage period.

Jankowska *et al.* (2012) [147] also studied the possibility of treat yogurt with HHP treatment (200 and 250 MPa, at 18 ^{o}C and 4 ^{o}C during 15 minutes) to its preservation and is was concluded that HHP treatment can extend the shelf-life of yogurt as well as improve its organoleptic properties. In addition, no significant differences in bacteria survival rate, antibacterial activity or acidity were found in pressurized yogurts. It was also observed that *L. bulgaricus* and *S. thermophilus* had a higher survival rate during the 4-week storage period following yogurt pressurization at 4 ^{o}C, when compared with non-pressurized yogurt. Acidity of both the pressurized yogurts at 18 ^{o}C and 4 ^{o}C remained at a similar level during storage period. Regarding to antibacterial activity towards pathogen test strains, yogurt pressurized at 4 ^{o}C showed a higher antibacterial activity than yogurt pressurized at 18 ^{o}C during the storage period. In addition, it was also verified differences in taste and smell between yogurts pressurized at 4 ^{o}C and 18 ^{o}C and controls one. Yogurt pressurized at 18 ^{o}C presented a better taste and smell. No differences were found in the appearance or consistency of both pressurized yogurts.

So, the application of hydrostatic pressure directly to yogurts has been proposed as an alternative to the use of additives, which can adversely affect the yogurt taste, flavor, aroma and mouth-feel [148].

II. OUTLINING AND PURPOSE OF THE WORK

It was verified that the studies about sub-lethal pressure stress response, principally fermentation under pressure, are scarce, as showed previously [135]. To the fermentative processes already made under pressure, it was verified that it can increase the fermentative rate, product yield and/or change the metabolism of fermentative microorganisms (production of different products at atmospheric pressure and at higher pressures). So, the application of this technology to food investigation can bring new characteristics to final product and/or fermentative process. Taking into account that the fermented products that are more popular among population are dairy products like yogurt, it would be interesting to study the effect of HHP in lactic acid fermentation, what it has not been studied yet. For that, it is expected that will be effects not only on the fermentation rate but also on the characteristics of final product, like modification of its organoleptic and nutritional proprieties.

Therefore, the purposes of this work are the study of the application of HHP technology on the yogurt production process and investigate the effect of HHP on microorganisms' growth and development, mainly on chemical and microbiologic characteristics of yogurt. For that, it was used set yogurt as inoculum and the effect of HHP was studied through several parameters. In order to monitor lactic fermentation, it was analyzed pH, titratable acidity and reducing sugars concentration, which give product and substrate concentration over time. So, with these parameters the fermentation rate under different conditions can be quantified. In addition to these analyzes, others were performed, such as concentration of D-glucose, D- and L-lactic acid, acetaldehyde and ethanol and a microbiologic analysis to the starters culture of set yogurt.

III. MATERIAL AND METHODS

1. YOGURT'S PRODUCTION

1.1. Sample Preparation

UHT (Ultra High Temperature) treated semi-skimmed milk from Auchan was inoculated with plain yogurt DANONE in a proportion of 80 mg of yogurt per mL of milk. After homogenization, the mixture was transferred to a heat sealed plastic bag with 8 cm × 2.5 cm dimension. To avoid sample contamination, the steps described below were performed in an aseptic environment, within a laminar flow cabinet.

1.2. Yogurt's Production

1.2.1. Fermentation under HHP

Lactic acid fermentation was carried at process optimal temperature (43^{o}C) under different HHP conditions. The experiments were executed in High Pressure System U33, Unipress Equipment, Poland, own by the Chemistry Department of University of Aveiro. This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature, using a mixture of propylene glycol and water as pressurizing fluid and to control the temperature in the external jacket. The pressure conditions tested were 5, 15, 30, 50 and 100 MPa, using as control, fermentation under atmospheric pressure (0.1 MPa), keeping all other parameters constant. The fermentation takes place during 600 minutes and over this time were collected several samples and, to stop fermentation, they were immersed in an ice bath and kept at 4 ^{o}C, or liquid nitrogen and stored at – 80 ^{o}C, until they were used for microbiological or for physicochemical analysis, respectively. Each experiment was performed in duplicate and the analyses carried out in triplicate.

1.2.2. Fermentation under Combined Pressure Conditions

In addition, it was performed a different type of experiments, in which pressure pre-treatments were applied before fermentation, keeping the temperature constant at 43 ^{o}C. In these experiments, the mixture was exposed to a HHP pre-treatment (50 or 100 MPa) for 90 or 180 minutes and then it was transferred to a bath at process temperature and

atmospheric pressure to continue the fermentation. Over the fermentation time at atmospheric pressure, several samples were collected and, to stop fermentation, they were immersed in an ice bath and kept at 4 ^{o}C, or liquid nitrogen and stored at – 80 ^{o}C, until they were used for microbiological or for physicochemical analysis, respectively. Each experiment was performed in duplicate and the analyses carried out in triplicate. The experimental design described in this section is represented in Table 1.

Table 1. Experimental design of fermentation under combined pressure conditions.

HHP pre-treatment		**P_{atm} fermentation**
HHP intensity (MPa)	**Time (minutes)**	**Time (minutes)**
50	90	0
		90
		360
100	90	0
		90
		210
		360
		510
		600
100	180	0
		90
		210
		360
		510
		600

2. YOGURT'S CHARACTERIZATION

2.1.Physicochemical Analysis

2.1.1. pH and Titratable Acidity

During lactic acid fermentation, one of the changes verified is the production of lactic acid, which causes a decrease of pH over the fermentation period. So, pH value is an easy-to-measure parameter and it is relevant to monitor the acids production during fermentation process. In this work, pH of the fermentative medium was measured using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Spain), at 25^{o}C.

It is important to determine also titratable acidity during fermentation monitoring. This parameter allows the calculation of total acid content in samples, through an acid-base titration and the obtained results are expressed in lactic acid concentration. This analysis was performed using a Titromatic 1S (Crison Instruments, S. A., Spain), accordingly to Chandan *et al.* [149] with some modifications: 1.50 mL of yogurt sample were diluted in 10.5 mL of water and then titrated with a 0.1N NaOH solution, until pH of 8.9 (the average phenolphthalein end point). The obtained results are expressed in g of lactic acid/L of yogurt.

2.1.2. Reducing Sugars Concentration

Other parameter used to monitor the fermentation is the concentration of reducing sugars that provides the substrate consumption rate during fermentation. In the fermentation medium, the main reducing sugars present in the sample are lactose, glucose and galactose, which are metabolized by the starter cultures over the fermentation time, leading to the production of lactic acid and other products.

To determinate the concentration of reducing sugars present in fermentation medium it was applied a colorimetric method using 3,5-dinitrosalicylic acid reagent (DNS), described by Miller, 1959 [150]. In this method, DNS reagent is prepared in an alkaline solution and reducing sugars, which have a free aldehyde or ketone group, are able to reduce the 3-5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, while the sugar's aldehyde group is oxidized to an aldonic acid, as represented in Figure 8. The formed acid

has an orange color and therefore solution intensity depends on acid formed content and thus depends on reducing sugars concentration [151].

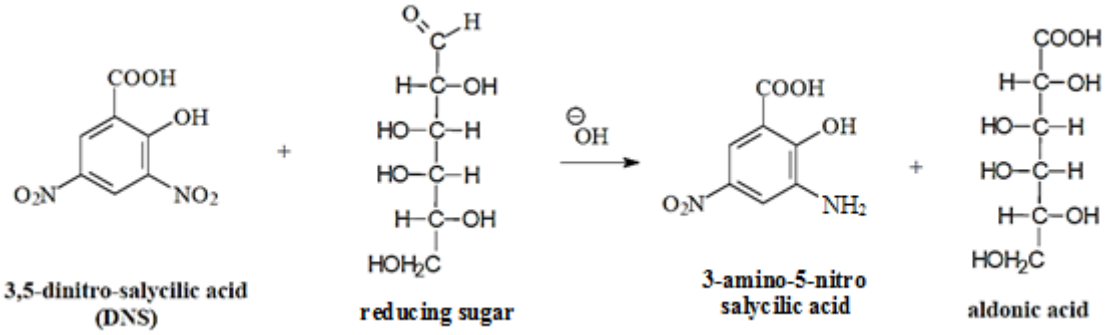

Figure 8. Reaction of reducing sugar with 3,5-dinitro-salycilic acid (DNS) reagent [151].

Therefore, for the reducing sugar concentration determination, 1.0 mL of DNS reagent (preparation described in Appendix I) was added to 1.0 mL of sample and then the mixture was placed in a boiling water bath during 5 minutes. After that time, the mixture was cooled in an ice bath (to stop the reaction), diluted with 10 mL of distilled water and then the absorbance was measured at 540 nm, in Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Thermo Fisher Scientific Inc., USA). The concentration values were calculated using a calibration curve, obtained from glucose standard solutions, and are expressed in g of reducing sugars/L of yogurt.

2.1.3. D-Glucose Concentration

D-glucose is not present in higher concentrations in milk, but is one of the main substrate involved in lactic acid fermentation. The major sugar present in milk is D-lactose, but this sugar suffers hydrolysis prior to the fermentation process, originating D-glucose and D-galactose. As stated previously, many starter cultures are not capable of galactose digestion and in consequence only glucose is used as substrate for fermentation.

In this work D-glucose was measured using the enzymatic test kit D-Glucose GOD-POD (AK00161) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. The principle of this method is described by the following reactions [152]:

D-glucose + O_2 + H_2O $\xrightarrow{GOD}$ D-gluconate + H_2O_2

2 H_2O_2 + *p*-hydroxybenzoic acid + 4-aminoantipirine $\xrightarrow{POD}$ quinoneimine dye + 4 H_2O

In a first stage, D-glucose is oxidized by glucose oxidase (GOD), producing D-gluconate and hydrogen peroxide and in the presence of peroxidase (POD) hydrogen peroxide is then oxidatively coupled with 4-aminoantipirine (4-AAP) and a phenolic compound (in this case *p*-hydroxybenzoic acid) to yield a red quinoeimine dye, with a maximal absorbance at 510 nm. So, the absorbance at 510 nm is quantitatively proportional to the concentration of glucose present in the sample [153].

To perform this analytical test, samples were first centrifuged (at 10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain D-glucose concentrations between 100 and 1000 mg/L. After the absorbance reading (λ=510 nm), D-glucose concentration was calculated using a respective calibration curve, taking into account the respective dilution, and the results were expressed in g of D-glucose/L of yogurt.

2.1.4. D-/L-Lactic Acid Concentration

As stated previously, during yogurt's production both lactic acid stereoisomers are produced (*S. thermophillus* produces L-lactic acid and *L. bulgaricus* synthesizes D-lactic acid). So, the determination of the two isomers concentration and respective proportion, can be used to infer the contribution of each starter culture to the fermentation.

In this work, D- and L-lactic acid concentrations were determined with an enzymatic test kit D-/L-Lactic acid (AK00141) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. With this test kit the assays for D-lactic and L-lactic acids are performed separately. The determination of D-lactic acid is based on the following two coupled reactions [154]:

D-Lactate + NAD^+ $\xrightarrow{D\text{-}LDH}$ Pyruvate + NADH + H^+

Pyruvate + D-Glutamate $\xrightarrow{D\text{-}ALT}$ D-Alanine + 2-Oxoglutarate

For that, the amount of NADH formed is measured, at 340 nm, through the combined action of D-lactate dehydrogenase (D-LDH) and D-alanine aminotransferase (D-ALT). There are two reactions because the first one is an equilibrium reaction and the second one will be necessary to combine in order to complete the reaction [154].

To determine L-lactic acid concentration, it is required a similar set of reactions but the oxidation to pyruvate by NAD+ is catalyzed by L-lactate dehydrogenase (L-LDH) instead, as follows [154]:

$$\text{L-Lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{D-Glutamate} \xrightarrow{\text{D-ALT}} \text{D-Alanine} + \text{2-Oxoglutarate}$$

To perform this analysis, samples were initially submitted to a centrifugation (at 10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain concentrations between 0.33 and 20 mg/L in the case of D-lactic acid and between 0.20 and 20 mg/L in the case of L-lactic acid. After the absorbance reading (λ=340 nm), concentration values were calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in g of D- or L-lactic acid/L of yogurt.

2.1.5. Acetaldehyde Concentration

As mentioned above, acetaldehyde is a carbonyl compound responsible for the typical flavor of yogurt, which is formed by lactic acid bacteria during fermentation period. So, the measurement of acetaldehyde concentration in yogurt samples obtained in this work show us the potential influence of the pressure treatment on yogurt taste and flavor.

Acetaldehyde concentration was determined using the enzymatic test kit Acetaldehyde (AK00051) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. The principle of this method is described by the following reaction [155]:

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{Acetate} + NADH + H^+$$

In this analysis, the amount of NADH formed is measured through the action of aldehyde dehydrogenase (Al-DH), which can be detected at 340 nm. Thus, the

absorbance's intensity is proportional to the amount of acetaldehyde in the sample volume [155].

To perform this test, samples were previously centrifuged (at 10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain acetaldehyde concentrations between the linearity limits, 0.25 and 10 mg/L. After the absorbance reading (λ=340 nm), acetaldehyde concentration was calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in mg of acetaldehyde/L of yogurt.

2.1.6. Ethanol Concentration

In this work, it was also measured the ethanol concentration, using an enzymatic test kit (AK00061) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. Ethanol determination by this analytical test kit is based on the following reactions [156]:

$$\text{Ethanol} + NAD^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + NADH + H^+$$

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{Acetate} + NADH + H^+$$

In this test kit, ethanol concentration is measured through the amount of NADH formed due the combined action of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (Al-DH), measured at 340 nm. So, the NADH formed is stoichiometric with twice the amount of ethanol in sample volume [156].

Before the analysis, samples were centrifuged (10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain ethanol concentrations between the linearity limits, 0.13 and 6 mg/L. After the absorbance reading (λ=340 nm), ethanol concentration was calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in g of ethanol/L of yogurt.

2.2.Microbiological Analysis

In this work, the effect of pressure on the viability of starter cultures is studied and for that it was performed a microbial count in samples fermented at different pressure conditions.

To perform the microbiological analysis, 1 mL of yogurt sample was transferred aseptically into a sterile tube with 9 ml of Ringer's solution and homogenized. Each sample was prepared in duplicate. Then serial decimal dilutions in sterile Ringer's solution were prepared and 1 mL samples of the appropriate dilutions were spotted on the plates, also in duplicate. To analyze the two starter cultures, the enumeration was carried out using a pour plate technique, but different selective media and incubation conditions were used according to the microorganism in question. After the incubation time, plates containing 15 to 300 colonies were enumerated, and the counts were expressed as $\log_{10}$ CFU/mL of yogurt.

2.2.1. *Lactobacillus bulgaricus* Count

The *L. bulgaricus* count was determined on double-layer agar plates of MRS (Lactobacillus Agar acc. de Man, Rogosa and Sharpe - Merck, Germany) medium, pH 5.7 ± 0.2, which was previously sterilized at 121^{o}C for 15 minutes. The cultures were then enumerated after incubation at 30 ^{o}C for 5 days [157].

2.2.2. *Streptococcus thermophillus* Count

The *S. thermophilus* count was carried out in M17 (Liofilchem, Italy) medium, pH = 7.2 ± 0.2, sterilized at 121^{o}C for 15 minutes. The inoculations were incubated at 37 ^{o}C for 72 h [147].

3. ACTIVATION VOLUME CALCULATION

Activation volume (Va) is, by definition, a quantity derived from the pressure dependence of the reaction rate constant [158], and its calculation is performed using Eq. 1:

$$\ln(k) = \ln(A) - Va \times \frac{p}{R_p \times T} \qquad (1)$$

where k is the reaction rate constant, A is a constant, V_a the activation volume (cm^3/mol), p is the pressure (MPa), R_p the universal gas constant (8.314 cm^3 MPa /(K mol)) and T is the absolute temperature (K). The activation volumes were calculated by linear regression analysis.

4. STATISTICAL ANALYSIS

In order to obtain the differences between the results obtained from different pressure conditions, it was tested at a 0.05 level of probability with the software STATISTICA 6.1 (Statsoft, Inc., Tulsa, OK). The effects of pressure level were tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey HSD) to identify the differences between samples.

IV. RESULTS AND DISCUSSION

1. PHYSICOCHEMICAL ANALYSIS OF LACTIC ACID FERMENTATION OF YOGURT

1.1. Monitoring Yogurt's Production under Pressure

In this section, the fermentation under a crescent range of pressures (5, 15, 30, 50 and 100 MPa) was monitored through analysis of product formation (titratable acidity) and substrate consumption (reducing sugars concentration) by lactic acid bacteria involved in yogurt's production. In addition, pH of the obtained samples was also analyzed, since this parameter is important to verify if the final product can be considered a yogurt, as stated previously [1]. Statistical analysis was also performed in this work in order to verify if differences between the analyzed samples are significant or not and the obtained results are present in Appendix II – section a).

The fermented samples at atmospheric pressure (0.1 MPa) were used as controls in each experiment, i.e. for each fermentation at a different pressure, the fermentation at atmospheric pressure with the other conditions being constant was also performed. In Figures 9, 10 and 11 are represented the lactic acid concentration, pH and reducing sugars concentration during fermentation time, under the different analyzed pressures, respectively. The results of fermentation at atmospheric pressure are indicated as the mean of all results obtained for this fermentation condition.

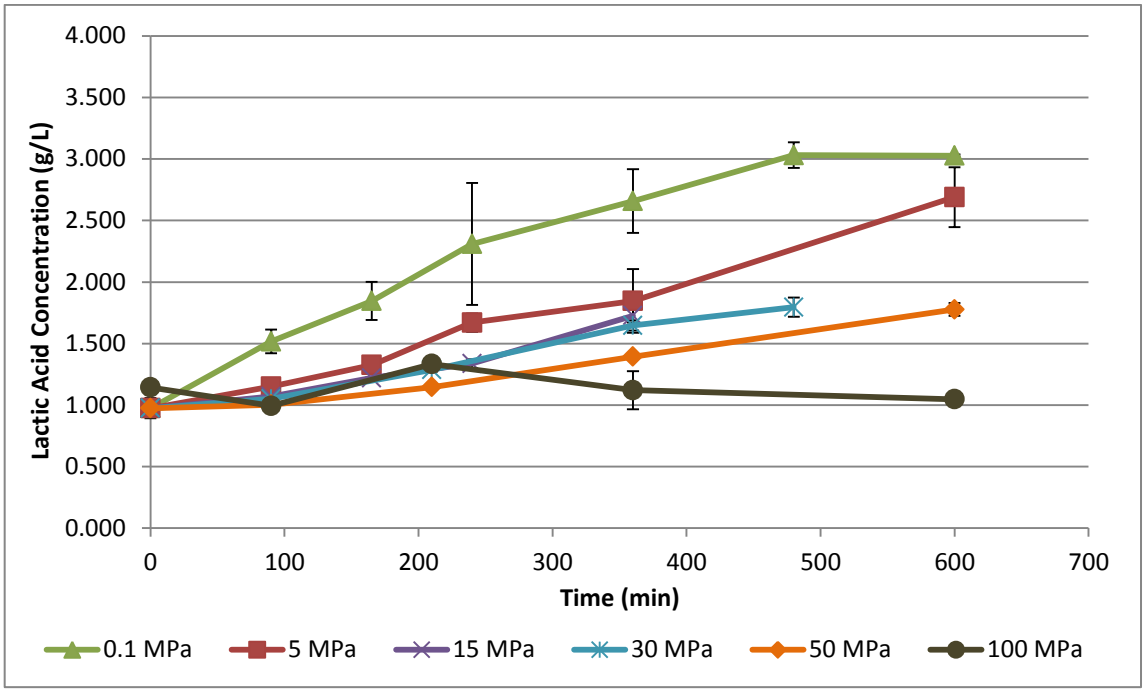

Figure 9. Lactic acid concentration during fermentation time, under different pressures (5-100 MPa). Fermentation at atmospheric pressure was used as control.

In Figure 9, the obtained results for titratable acidity are presented and they are expressed as lactic acid concentration, the main product of lactic acid fermentation. Analyzing the control fermentation, it is possible to note that it has a profile of product formation during fermentation time – in the beginning lactic acid concentration increase and in the end it starts to stabilize around 3.000 g/L of lactic acid.

With the increase of fermentation pressure, it was verified that the lactic acid production decrease significantly, until at 100 MPa there was no lactic acid production during 600 minutes of fermentation, because the initial and final lactic acid concentrations are similar (≈ 1.000 g/L). In addition, it is possible to note that at the end of fermentation at 5 MPa, the lactic acid concentration is similar to control's final concentration ($p > 0.05$), approximately 3.000 g/L. So, although fermentation under this pressure is slower, after 600 minutes (10 hours) in these conditions, a similar fermentation level than in the control was reached. Thus it is possible to point out that increasing pressure influences the rate of fermentative process through its gradual inhibition and higher pressures, namely 100 MPa, are able to stop lactic acid fermentation. This can be explained by a possible disruption of

the cellular function and even viability when cells are exposed to this range of pressures [86].

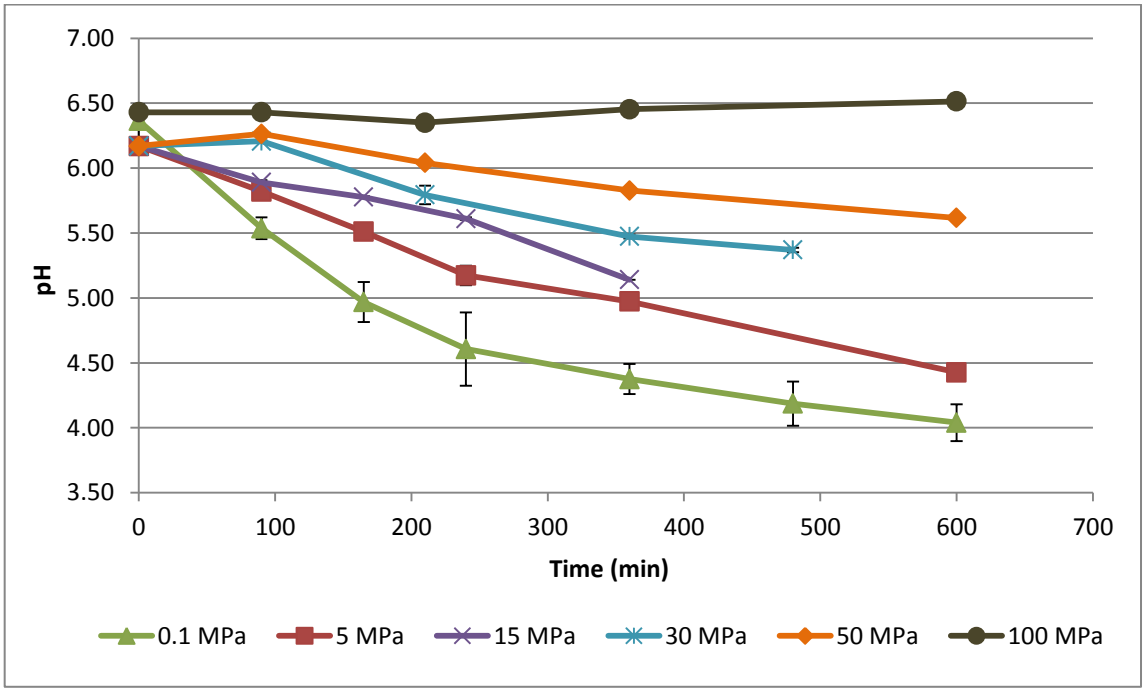

Figure 10. pH variation during fermentation time, under different pressures (5-100 MPa). Fermentation at atmospheric pressure was used as control.

Figure 10 represents the obtained pH variation during fermentation at the tested pressures in this work. This parameter is related to acid formation, so in this case pH can be related to lactic acid production represented at Figure 9, since the increase in lactic acid concentration converts in decrease of pH (increase of acidity). Therefore, analyzing the obtained results, it is possible to note that, in the control, pH varies according to results expected during a yogurt's fermentative process and by analyzing lactic acid production (Figure 9). So, the fermentation starts with a high pH decrease rate, which is concordant with lactic acid formation. Then pH variation rate decreases, as detected for lactic acid concentration in this case. As stated previously, pH is an important parameter to define the end of fermentation, because industrial process is stopped when it is reached pH 4.5 [1] – approximately casein's isoelectric point [1] – and then the yogurt is finished. Taking this into account it was verified that at atmospheric pressure, yogurt was obtained after approximately 300 minutes (5 hours) of fermentation, which takes longer than

fermentation time utilized in industrial process – 2-3 hours [1, 3]. This time difference can be explained by slight differences in fermentation conditions between laboratory and industrial scale and/or differences in starter cultures' content and concentration.

In Figure 10, the pH variation during fermentation under different pressures is also represented and it is verified that the pressure increase leads to a decrease on pH variation rate, as previously observed to lactic acid production rate. Thus, the final pH of each represented fermentation increases with increasing pressure, which means that with pressure there is inhibition of the fermentative process. In fermentation at 100 MPa, pH remains at approximately 6.50, without significant variation, during the whole fermentative process, which suggests that fermentation is completely inhibited at this pressure range. These results are again in concordance with results obtained for lactic acid production (Figure 9).

Furthermore, it is verified that at 5 MPa the fermentation has a similar profile as the control, but pH variation is slower. Despite this, after 600 minutes (10 hours) under 5 MPa, the sample reached pH 4.43, thus yogurt as final product (pH 4.5) is obtained. So, the fermentation under 5 MPa can have as final product a yogurt, despite fermentation time is twice longer than the process at atmospheric pressure.

The parameters analyzed above provide information about the product formation during fermentation time, but to complement this information, it was necessary also study the substrate consumption profile during that time, to understand the several fermentative processes performed in this work. For that, the reducing sugars concentration during fermentation time was analyzed and the obtained results are represented at Figure 11. This analysis can bring the information necessary about substrate consumption in lactic acid fermentation of yogurt, because the sugars present in milk and that are metabolized by starter cultures (lactose, glucose and galactose) have reducing power.

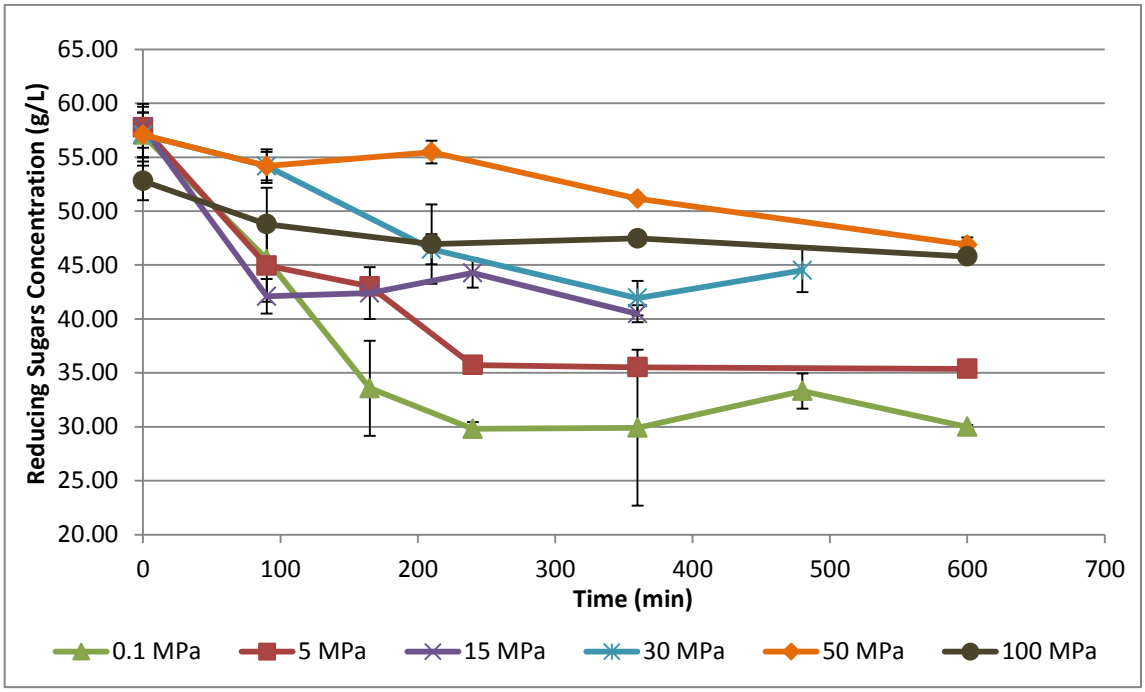

Figure 11. Reducing sugars concentration during fermentation time, under different pressures (5-100 MPa). Fermentation at atmospheric pressure was used as control.

Analyzing the obtained results for reducing sugars concentration during fermentation time, it is possible to note that the fermentation used as control (at atmospheric pressure) has a substrate consumption profile typical of fermentative processes. In the beginning of fermentation there is a higher substrate consumption rate until stabilization around 240 minutes (4 hours), approximately the time necessary to obtain a yogurt (Figure 10). After this time, the sugars concentration remain constant (≈ 30.00 g/L) until the end of fermentation time. Therefore, this substrate profile is concordant with results obtained previously for product formation, since when there is a higher product formation rate, there is more substrate consumption.

For samples fermented at the others analyzed conditions, it is possible to note that the increasing of pressure leads to a higher final concentration of sugars, which indicates that there is a decrease of substrate consumption rate by starter cultures. At 5 MPa, the final concentration of sugars is approximately 35.00 g/L, which is higher that the final sugars concentration in control fermentation ($p < 0.05$).

Although it was previously verified that, at 100 MPa, fermentation does not occur, in this analysis there was a decrease in substrate concentration (from 52.80 to 42.79 g/L).

This decrease indicates that there was substrate consumption by starter cultures, mainly in the beginning of fermentation, which can be interpreted as a possible initial adaptation of microorganisms to pressure. But analyzing all the parameters used to monitor the fermentation (lactic acid concentration, pH and reducing sugars concentration), it can be pointed out that this adaptation is not enough for microorganisms to overcome the pressure stress and carry out the fermentation.

In addition, the results previously showed for 90 and 360 minutes were plotted versus pressure, in order to try to better elucidate the effect of pressure on the fermentation process (Figure 12, 13 and 14 for lactic acid concentration, pH and reducing sugars concentration, respectively).

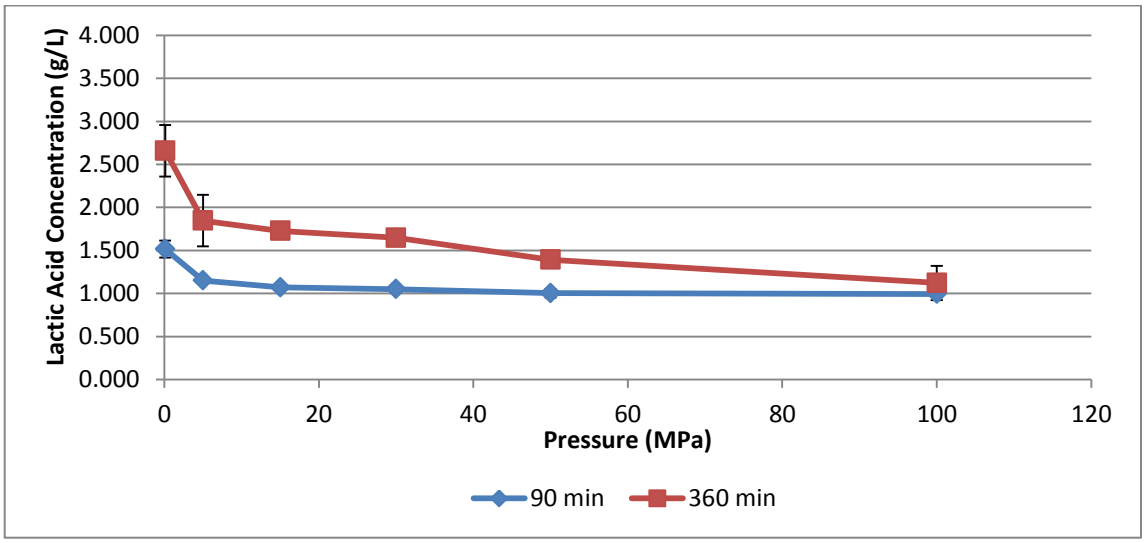

Figure 12. Lactic acid concentration, after 90 and 360 minutes of fermentation, in function of pressure.

In Figure 12 are represented the lactic acid concentration, in the two chosen times, in function of the increasing pressure. As verified previously, with increasing of pressure there is a lower lactic acid production due to fermentation inhibition.

At 90 minutes, the difference between the values of samples fermented at atmospheric pressure (0.1 MPa) and at 100 MPa is low. This can be explained for these samples are in an initial phase of fermentation, so even at atmospheric pressure, the time of fermentation is short for a high lactic acid formation. Thus, with pressure increase it was

verified that the lactic acid concentration tends to stabilize around the initial value of concentration (≈ 1.00 g/L – Figure 9). Thus it means that with pressure increase there was fermentation inhibition after 90 minutes, even for pressures as low as 30 MPa, where the lactic acid concentration is already similar to the initial value (0 minutes).

The other point of time analyzed, 360 minutes of fermentation, represents an advanced stage of fermentation. For that, it was verified that, between atmospheric pressure and 100 MPa, there is a significant difference between the respective values of lactic acid concentration. This can be explained by fermentation inhibition with the increase of pressure, which is total at 100 MPa. For this pressure, the value of the two analyzed points is similar, which in turn is similar to lactic acid concentration in beginning of fermentation, as expected due to complete fermentation inhibition in these conditions.

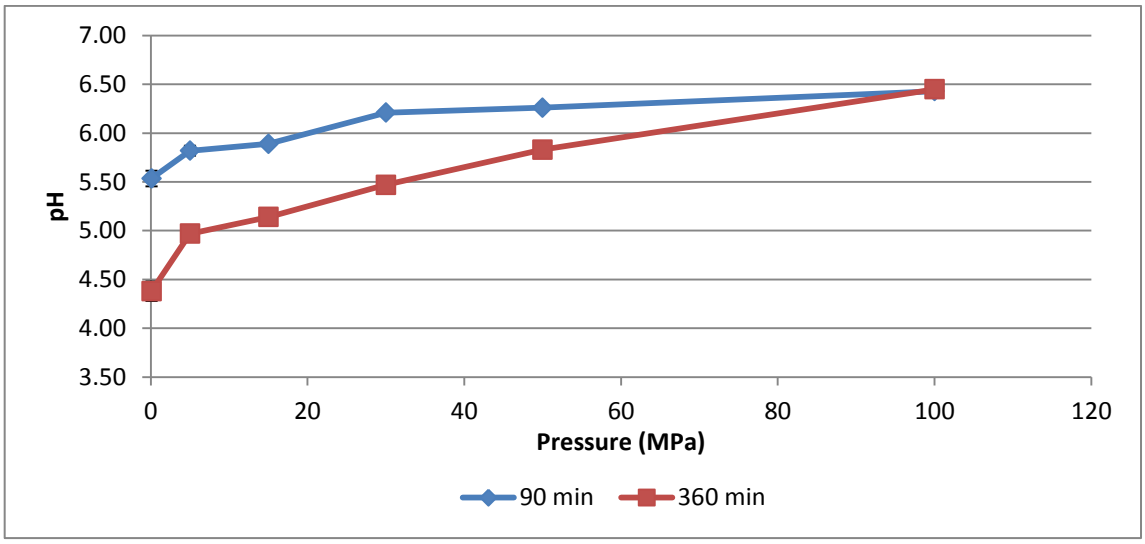

Figure 13. pH variation, after 90 and 360 minutes of fermentation, in function of pressure.

Making the same analysis for pH variation, the results obtained are represented in Figure 13, where it is verified, once again, that pressure has an inhibitory action in lactic acid production by fermentation, since pH increases (acidity decreases) with the pressure increasing. In addition, analyzing Figure 13, it is also verified that, at the initial phase of fermentation (90 minutes), pH variation with pressure increase is lower than after 360 minutes of fermentation. Furthermore, in fermentation under 100 MPa, it can be observed that fermentation does not occur because the values after 90 and 360 minutes of

fermentation are similar. In conclusion, these results are in accordance with the previous results, since a lower lactic acid formation leads to a lower acidity (lower decrease of pH).

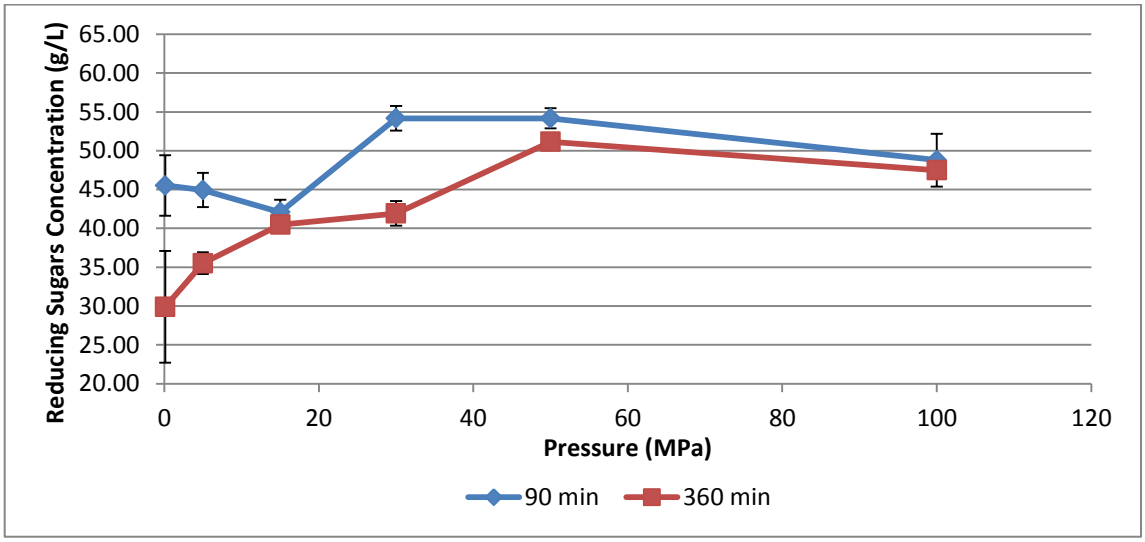

Figure 14. Reducing sugars concentration, after 90 and 360 minutes of fermentation, in function of pressure.

In Figure 14, the reducing sugars concentration after 90 and 360 minutes of fermentation is represented in function of pressure increase. In this case, the point at 15 MPa after 90 minutes of fermentation reveals some possible experimental problems, which can be explained by several factors, like problems with sample, with processing and/or even with analysis of reducing sugars determination. Apart from that, the results are in accordance with the previous results, because pressure increasing leads to an increase of reducing sugars concentration. These results indicate once again that the fermentation inhibition by pressure.

Therefore, by analysis of these results it is possible to conclude that pressure influences negatively the fermentative process, leading to its inhibition. This conclusion was showed by all physicochemical analyses used to monitor the yogurt production under pressure (titratable acidity, pH and reducing sugars concentration).

1.1.1. Activation Volume Calculations

Taking into account the results analyzed previously, it was performed a kinetic analysis, where it was calculated the Va for the parameters used to monitor fermentation,

such as titratable acidity, pH variation, which was translated in H^+ concentration, and sugars concentration. The obtained Va are presented in Table 2 and its detailed calculation are explained in Appendix III.

This kinetic parameter is important to extrapolate if one reaction is activated or not by pressure influence, since if Va is positive, the reaction is inhibited by increasing pressure, and vice-versa. Thus, after Va calculation for a certain reaction, it is possible to know if the reaction is retarded or not when pressure is applied to the reaction system. In addition, the higher the Va, the higher the effect of pressure on the reaction.

Table 2. Activation volume obtained for three parameters analyzed to monitor fermentation under pressure.

Parameter analyzed	Activation volume (Va)	r^2
Titratable Acidity	32.98 $cm^3.mol^{-1}$	0.94
H^+ concentration	66.33 $cm^3.mol^{-1}$	0.97
Sugars concentration	86.47 $cm^3.mol^{-1}$	0.82

Analyzing previous works in this area, it was verified that this kinetic analysis has never been carried out for any fermentative process under pressure. Only one work reported reaction rate constants for ethanol formation during fermentation, but no Va value was calculated [133].

Through analysis of Va values obtained for the evaluated parameters, it can be noted that they are positive, thus it is possible to conclude that the reactions analyzed are inhibited by pressure. So, these values are in accordance with conclusions obtained previously, that pressure influences negatively lactic acid fermentation. By analysis of Va value for each parameter, it is possible to know the level of the inhibition or activation of the respective reaction and/or reactions set, depending on analyzed parameter. Since in this case processes derived from fermentative process were analyzed, the obtained Va values are a global result of the metabolic reactions set involved in each parameter determination. Therefore, the reaction with a higher Va will be directly influence the Va value for reactions set, i.e. the final Va, since the whole process is limited by this reaction.

By Table 2, it can be concluded that the pressure most affected parameter for this case is substrate consumption, due to a higher Va of the three parameters. pH variation, i.e.

H^+ concentration during fermentation time, has an intermediate Va between the other two analyzed parameters and lactic acid production obtained by titratable acidity has a lower Va value, which indicates that this parameter is less affected by increasing pressure. It is important to note that the latter two parameters are related to each other, since the increase of titratable acidity usually leads to increase of acidity in a proportional way and consequently there is a pH decrease. Therefore, these results indicate that titratable acidity is less affected by pressure than pH, which can be explained by production of other acids, in addition to lactic acid, during the fermentative process. But these other acids produced do not change pH, but change the titratable acidity, since the capacity of acids to change the pH depends of their acidity constant (pKa), i.e. acids with lower pKa have a higher capacity to change pH and vice-versa. Therefore, analyzing the obtained results, it can be concluded that the produced acids profile during fermentation might be modified by pressure influence. Thus, indicates possible changes in metabolism. But, in order to reach a correct conclusion about these differences of acid production is necessary to perform a detailed analysis to acids profile of the obtained samples.

1.2. Monitoring of Yogurt's Production under Combined Pressure Conditions

In addition, it was also performed another type of experiment, consisting in the use of pressure pre-treatment before fermentation at atmospheric pressure (different pressure conditions over experiment time). For that, a pre-treatment with pressure was executed in a first phase and then the samples were transferred to a bath at atmospheric pressure (0.1 MPa), keeping the process temperature (43 ^{o}C) during the two phases. To monitor this fermentation, the analyzed parameters are the same than in the previous fermentation type (titratable acidity, pH variation and reducing sugars concentration). This experiment has as main purpose to verify if after pre-treatment with pressure is possible to obtain yogurt as final product during the subsequent fermentation at atmospheric pressure and if the final product and/or fermentative process has novel characteristics. In addition, when possible, a statistical analysis to analyzed samples was performed in order to verify the significance of the differences between them and the obtained results are present in Appendix II – section b).

In first represented case, the samples were pre-treated during 90 minutes under 50 MPa and in Figures 15, 16 and 17 are represented the lactic acid production, pH variation and reducing sugars concentration, respectively. In order to compare results, the fermentation profile at atmospheric pressure and 50 MPa are also represented.

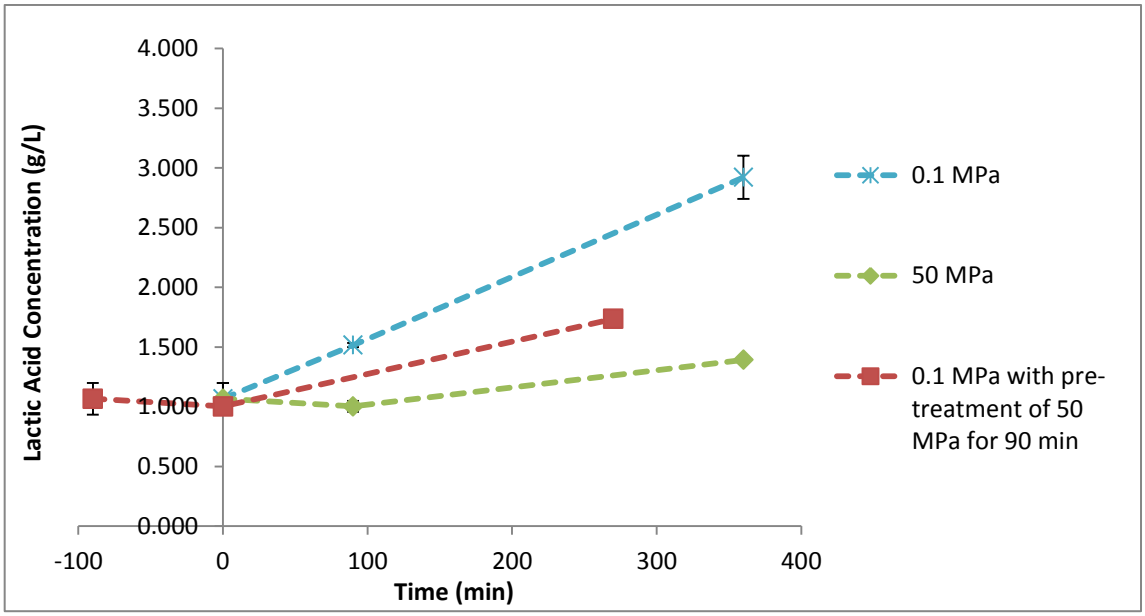

Figure 15. Lactic acid concentration during fermentation time, with a pre-treatment of 50 MPa for 90 minutes. To compare results, fermentation at atmospheric pressure and 50 MPa are also represented.

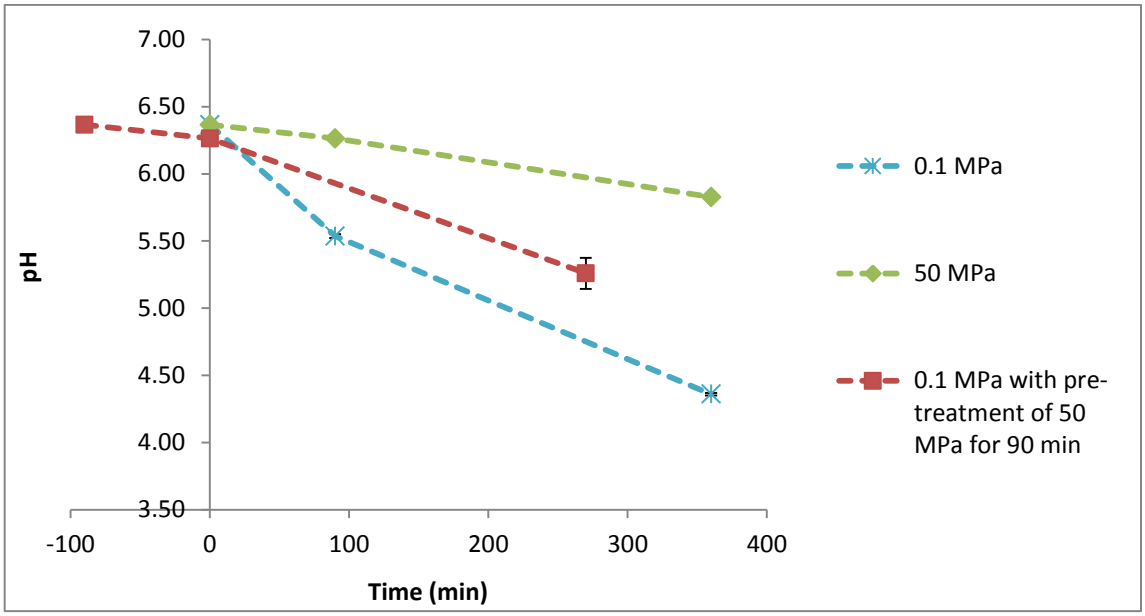

Figure 16. pH variation during fermentation time, with a pre-treatment of 50 MPa for 90 minutes. To compare results, fermentation at atmospheric pressure and 50 MPa are also represented.

During pre-treatment of 50 MPa for 90 minutes, both lactic acid concentration and pH remains apparently constant, which points out that fermentation does not occur or is low throughout this time. After pre-treatment, the samples are transferred to a bath at atmospheric pressure and it is possible to note that the two analyzed parameters varying in a faster manner (lactic acid concentration increase and pH decrease) than it would if remains at 50 MPa during all the time (360 minutes of fermentation). These results indicate that although in the pressure pre-treatment apparently fermentation does not occur, after that time at atmospheric pressure, the fermentative process occurs because there was lactic acid production. This behavior can be explained by an inhibition reversible of starter cultures activity during pre-treatment, because after that they are no longer under stress and are able to ferment. Since the rate of acid formation after pre-treatment is not similar to those that have been at atmospheric pressure over the whole fermentation time, it can be concluded that 90 minutes under this range of pressure influenced negatively the starter cultures, namely the lactic acid production.

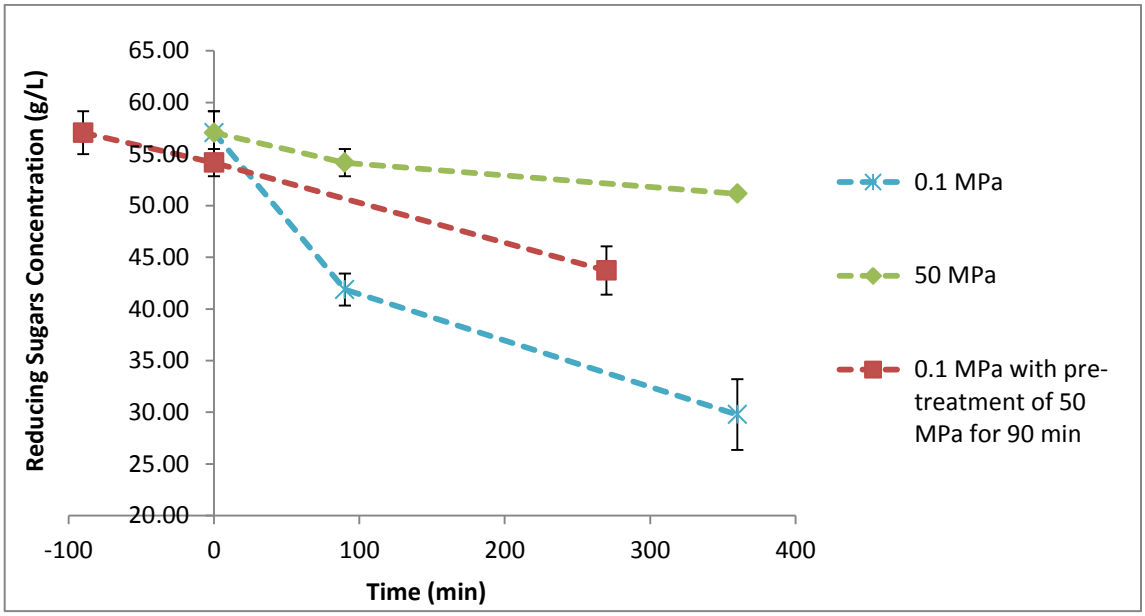

Figure 17. Reducing sugars concentration during fermentation time, with a pre-treatment of 50 MPa for 90 minutes. To compare results, fermentation at atmospheric pressure and 50 MPa are also represented.

In Figure 17, the reducing sugars concentration over time is represented and the obtained results are in accordance with previous ones. During pre-treatment, there is a decrease in sugars concentration, which points out that despite fermentation at these conditions does not occur, bacteria are present in medium and they can be consuming sugars to retain their activity or to adapt to this extreme conditions. When the fermentation conditions change to atmospheric pressure, it is verified that, apparently, the substrate consumption occurs at the same rate than during pre-treatment. But it is important to note that fermentation at atmospheric pressure of pre-treated samples was only monitored in the beginning and at the end (0 and 270 minutes), so it is impossible to infer the samples behavior during that time.

When comparing the substrate consumption of the pre-treated samples with the others results presented, it is possible to note that substrate consumption rate of pre-treated samples is lower than samples fermented at atmospheric pressure without pre-treatment, but higher than those fermented under 50 MPa. These results are in accordance with the results obtained to product formation (Figure 15 and 16), since it was verified that when samples are transferred to atmospheric pressure medium, despite there was fermentation, this process occurs in a slower manner than samples without pre-treatment.

In conclusion, during pre-treatment there was no fermentation, but after that the fermentation occurs in an intermediate rate between atmospheric pressure and 50 MPa. Meanwhile, there was substrate consumption during and after pre-treatment, in the latter, also in an intermediate manner between 0.1 MPa and 50 MPa, as the others analyzed parameters. So, it can be hypothesized that during pre-treatment some of cells present in medium are not able to withstand pressure and die, and the others are only inhibited with pressure during this time.

Furthermore, others experiments with combined pressure conditions were performed, where only changes the pre-treatment conditions. In this case, the pre-treatment was 90 minutes under 100 MPa and the respective lactic acid production, pH variation and reducing sugars concentration were measure during fermentation time, being represented in Figures 17, 18 and 19, respectively. The results for these parameters were compared to those of fermentation profile at atmospheric pressure and 100 MPa during the whole process time, that are also represented.

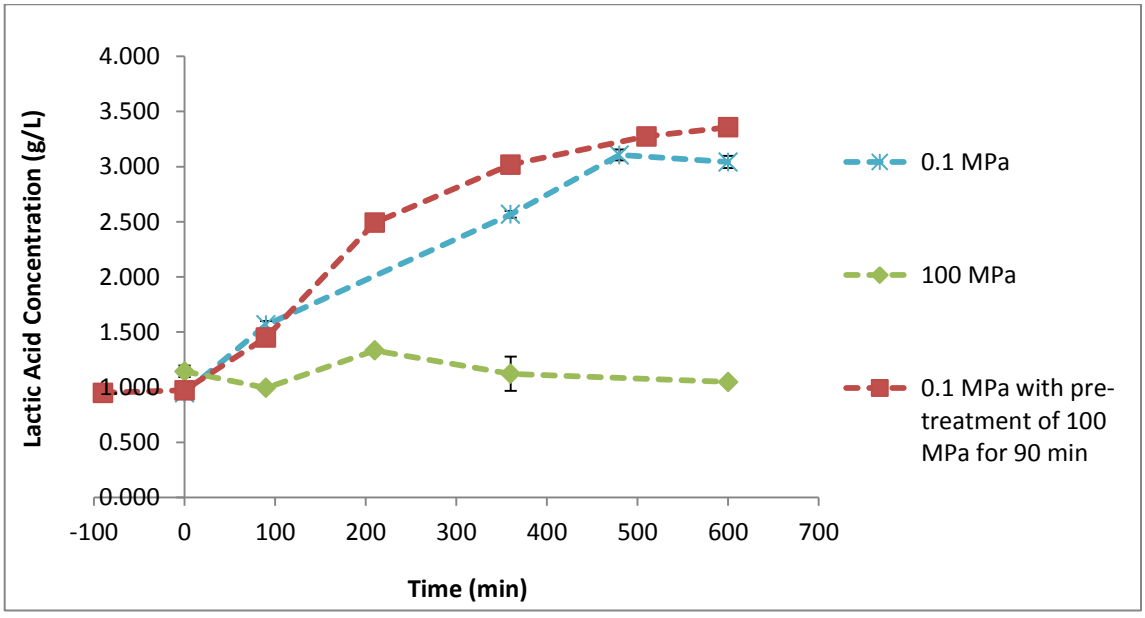

Figure 18. Lactic acid concentration during fermentation time, with a pre-treatment of 100 MPa for 90 minutes. To compare results, fermentation at atmospheric pressure and 100 MPa are also represented.

As previously stated, the fermentation is inhibited at 100 MPa which is reflected in the obtained results, since titratable acidity remains constant over the fermentation time. Therefore, during the pre-treatment performed in this experiment, it is verified once again that the fermentation inhibition at this range of pressures, since apparently there was no change in lactic acid concentration during this time. But, when the samples were transferred to a bath at atmospheric pressure, it was possible to note a marked increase in lactic acid concentration, reaching, after 150 minutes, higher values than fermentation only at atmospheric pressure (Figure 17). After 600 minutes of fermentation at atmospheric pressure, the lactic acid concentration reached by samples with pre-treatment is slightly higher than ones no pre-treated (3.357 g/L and 3.042 g/L, respectively, $p < 0.05$). So, despite samples with pre-treatment have a higher lactic acid production rate, in final phase of fermentation there is a stabilization of lactic acid concentration in both pre-treated and no pre-treated samples at similar acid concentration. This stabilization in both samples types can be explained by product inhibition in fermentation, i.e. the fermentation medium has already an acid concentration that microorganisms are not able to growth and subsequently perform lactic acid fermentation.

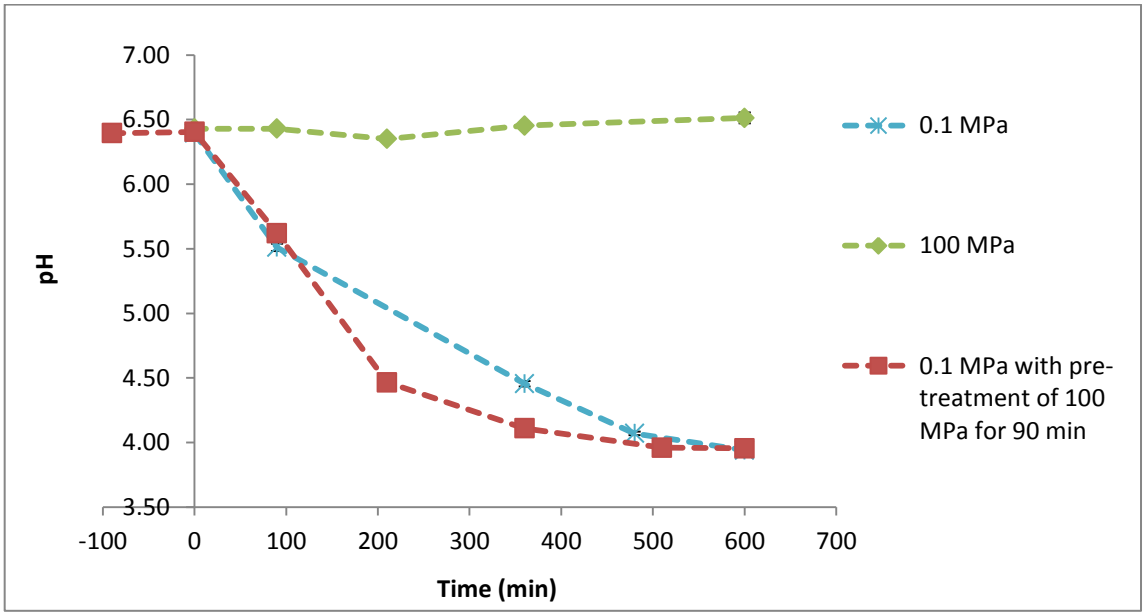

Figure 19. pH variation during fermentation time, with a pre-treatment of 100 MPa for 90 minutes. To compare results, fermentation at atmospheric pressure and 100 MPa are also represented.

pH variation during fermentation time of this experiment are represented in Figure 19. Analyzing the obtained results, it is possible to note that pH variation is in accordance with the obtained results for lactic acid production (Figure 18), since increase of lactic acid concentration leads to an acidity increase which is reflected as pH decrease. In this analysis, during pre-treatment, it is also verified that pH does not vary, which indicates once again the fermentative process inhibition due to employed pressure.

After samples transference to atmospheric pressure, there is a marked decrease of pH, as occurs with lactic acid concentration. So, in this analysis it is also verified that after pre-treatment, the fermentation rate is enhanced and this rate is higher than samples fermented at atmospheric pressure without pre-treatment. Taking into account that when it is reached pH 4.5 the fermented samples can be already considered yogurt and this is one of the main parameters to end fermentation in industrial process, it is possible to note that samples with this pre-treatment reaches that value after 210 minutes and samples without pre-treatment only reaches after 360 minutes of fermentation.

Therefore, we can conclude that the pre-treatment of 100 MPa during 90 minutes influences positively the fermentation rate and is obtained a yogurt as final product in a faster manner when this pre-treatment is applied to samples. Furthermore, at the end of 600 minutes of fermentation at atmospheric pressure, the both type of samples (with and without pre-treatment) reaches the same pH value (pH 3.96, $p > 0.05$).

In conclusion, the obtained results for lactic acid production and pH variation indicate that pre-treated samples have a higher fermentation rate during the subsequent fermentation at atmospheric pressure. This can occur because of some changes in cell metabolism due to the need that cells have to adapt to pressure during pre-treatment that they are subjected. And these changes lead to an increase of fermentation rate, when the samples are transferred to a bath at atmospheric pressure.

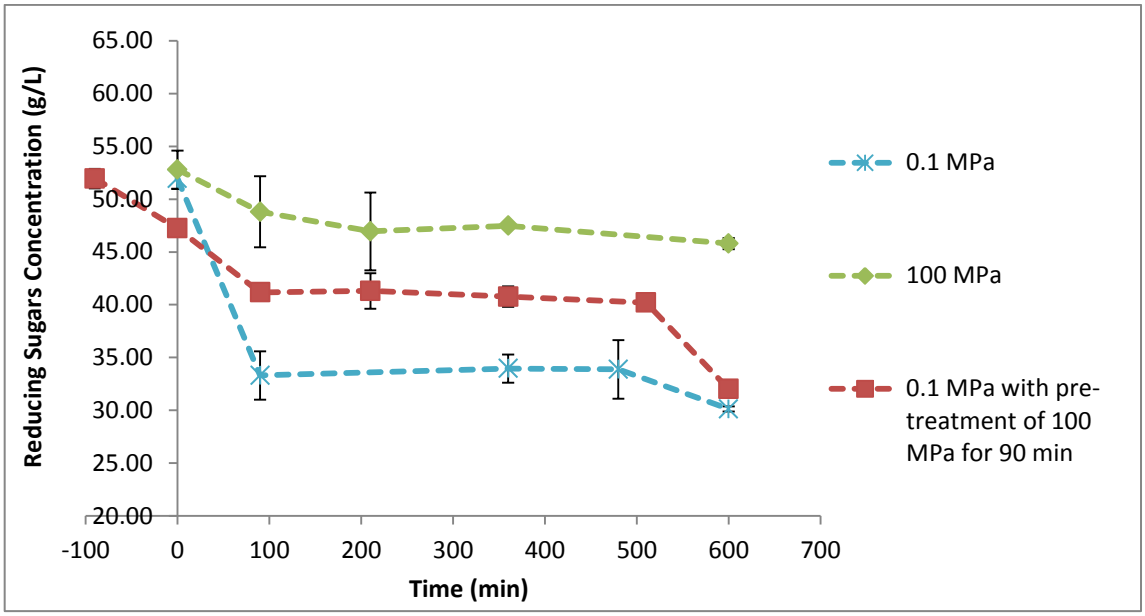

Figure 20. Reducing sugars concentration during fermentation time, with a pre-treatment of 100 MPa for 90 minutes. To compare results, fermentation at atmospheric pressure and 100 MPa are also represented.

For this pre-treatment, the cells substrate consumption was also analyzed, which is presented in Figure 20. By analysis of the obtained results it is possible to note that despite fermentation does not occur at 100 MPa, there is substrate consumption, mainly in the initial phase, which can be due to an attempt by microbial cells to adapt to pressure and hence they needed energy for that.

Therefore, during pre-treatment, there is substrate consumption, i.e. decrease of reducing sugars concentration, as represented in Figure 20. Furthermore, when samples are transferred to atmospheric pressure medium, there is still substrate consumption, which is in accordance with previous results, since fermentation occurs. But, after 90 minutes of fermentation at atmospheric pressure, the substrate consumption stabilizes until the end of fermentation, where there was a marked decrease in reducing sugars concentration, reaching a concentration similar to samples without pre-treatment, but significantly different ($p < 0.05$). These values can be due to an error in analysis, such as presence of interfering substances in samples and/or experimental errors during analysis performance, because the others analyzed parameters for these samples indicated that fermentation is occurring in a high rate. Thus it was expected that the sugars consumption followed the high rate of lactic acid production, which it is not verified.

Thus, it is possible to conclude that this pre-treatment can bring an advantage to yogurt production process, namely the increase of fermentation rate after pre-treatment of 100 MPa for 90 minutes. Furthermore, this can indicate that this range of pressure can be used as a storage method for milk and starters at fermentation temperature, because there was no fermentation at these pressure conditions and when the samples are depressurized, fermentation takes place, in even at a higher rate than samples fermented at atmospheric pressure during the whole process.

In order to evaluate the possible novel pressure application to milk and yogurt starters described above, a new experiment with a longer pre-treatment was performed, to verify if samples remain with the same behavior as the ones previously described. The obtained results are represented at Figure 21, 22 and 23, for titratable acidity, pH variation and reducing sugars concentration, respectively.

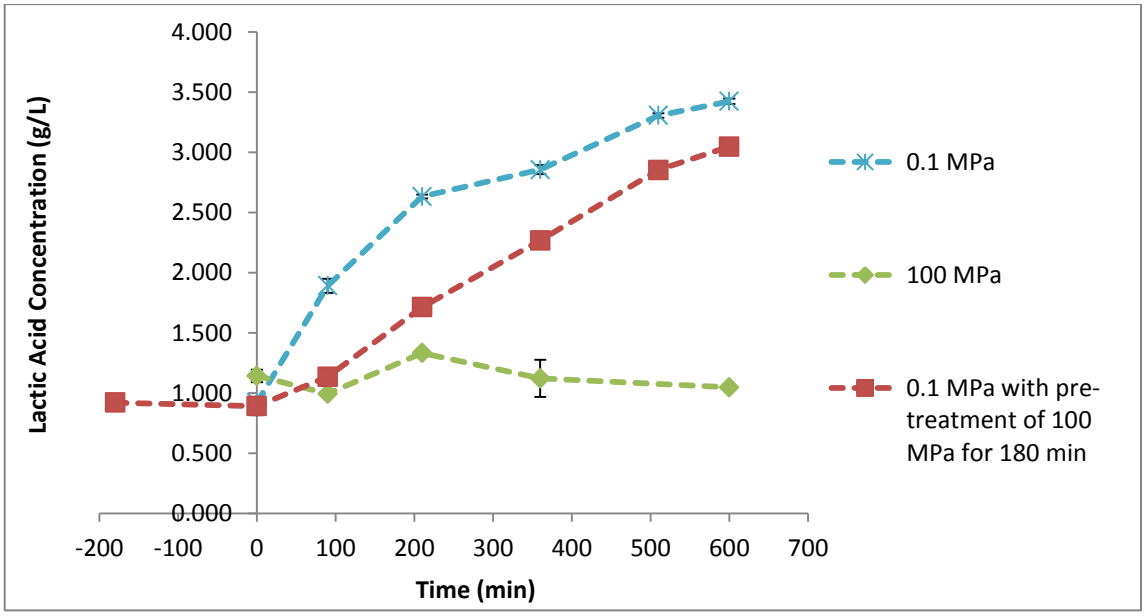

Figure 21. Lactic acid concentration during fermentation time, with a pre-treatment of 100 MPa for 180 minutes. To compare results, fermentation at atmospheric pressure and 100 MPa are also represented.

For the pre-treatment longer described above, the obtained results for titratable acidity are represented in Figure 21. By its analysis, it is possible to note that the titratable

acidity does not change during pre-treatment time, due to fermentation inhibition by HHP, as expected and previously observed. However after pre-treatment, fermentation occurs, since when the samples are transferred to an atmospheric pressure bath there is an increase on lactic acid concentration. But it is possible to verify that the lactic acid production rate is lower than the samples without pre-treatment and therefore lower than samples pre-treated during 90 minutes (Figure 18). Despite this, after 600 minutes at atmospheric pressure, samples with pre-treatment reached a slightly lower lactic acid concentration than samples no pre-treated (3.047 g/L and 3.423 g/L, respectively, $p < 0.05$). This can be explained by the increase of lactic acid concentration during the whole time in the first case and on the other case, there was a decrease in lactic acid production rate in the final phase of fermentation (due to product inhibition), leading to values approach.

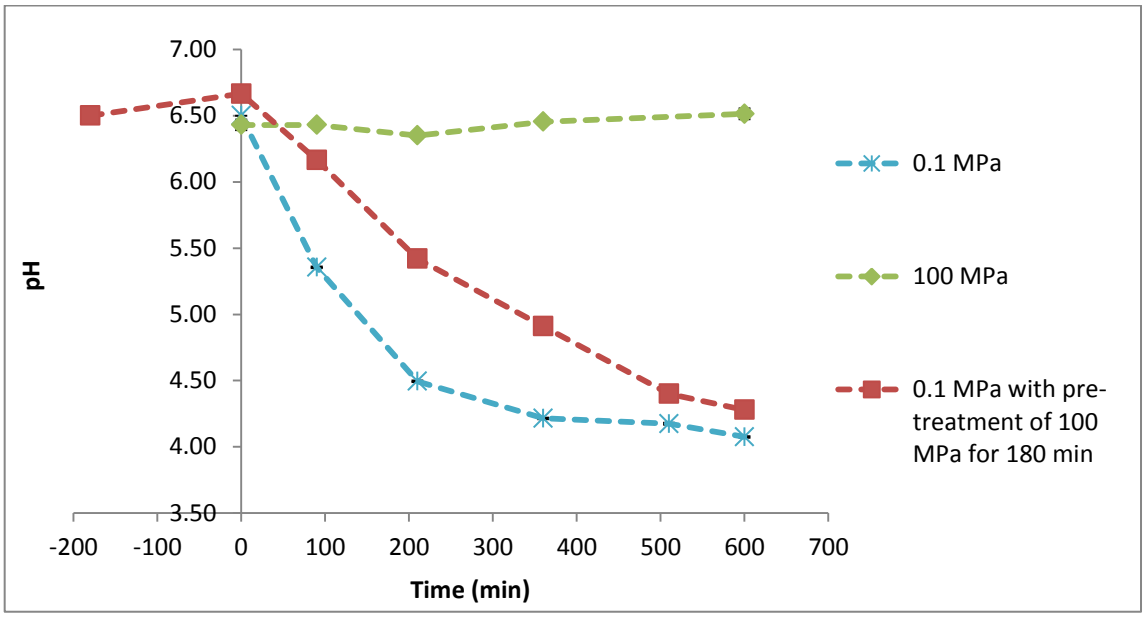

Figure 22. pH variation during fermentation time, with a pre-treatment of 100 MPa for 180 minutes. To compare results, fermentation at atmospheric pressure and 100 MPa are also represented.

In Figure 22 are represented the results of pH variation during this new experiment and it can be noted that this results are in accordance with the results previously described for lactic acid production (increase in acidity leads to decrease in pH values). During pre-treatment there is a slightly increase of pH, which indicates once again the fermentation inhibition, since during fermentation there is a decrease in pH and not an increase. After

pre-treatment, the fermentative process occurs due to pH variation, but it is important to note that pH variation have the same profile than titratable acidity (Figure 21), i.e. at atmospheric pressure, pH decrease rate for samples with pre-treatment is lower than for samples without pre-treatment. Despite that, pH value for pre-treated samples, after 600 minutes of fermentation at atmospheric pressure, is slightly higher than ones no pre-treated (4.28 and 4.08, respectively, $p < 0.05$), as previously verified in titratable acidity. Furthermore, it is possible to note that in the end of fermentation is possible to obtain yogurt as final product, since the obtained pH is lower than pH 4.5 – the required pH to stop lactic acid fermentation in industrial production process.

The fact that fermentation occurs slower in this case can be explained by cell death and/or irreversible inhibition during that longer pre-treatment, which indicates that the longer pre-treatment is, the slower is fermentation afterwards. Therefore, the pressure application for storage of milk and starter cultures suggested previously cannot be suitable, since pressure can influences negatively the starter cultures number.

Furthermore, for both pre-treatments used in this work (90 and 180 minutes), it is possible to point out that after pre-treatment, the fermentation, i.e. acid production, begins immediately after samples are transferred to atmospheric pressure, without any adaptation time to new pressure conditions. This may indicate that despite during pre-treatment, starter cultures attempt to overcome the pressure to perform fermentation, the adaptation mechanisms used can also operate at atmospheric pressure.

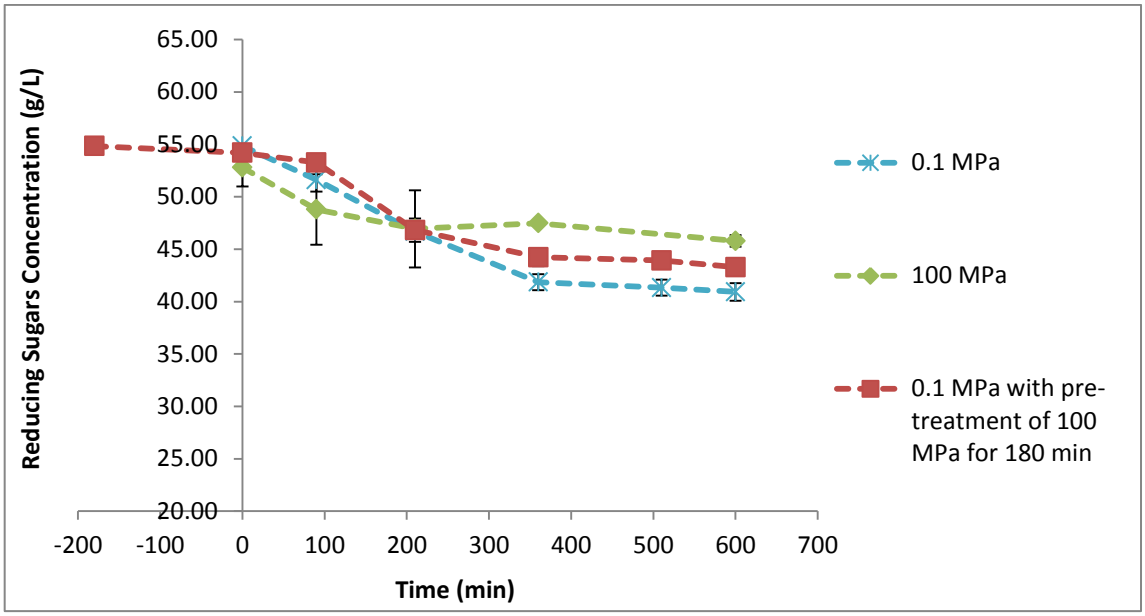

Figure 23. Reducing sugars concentration during fermentation time, with a pre-treatment of 100 MPa for 180 minutes. To compare results, fermentation at atmospheric pressure and 100 MPa are also represented.

In Figure 23 is represented the substrate consumption by starter cultures during this experiment. During the pre-treatment, it is possible to point out that apparently there was no decrease in reducing sugars concentration, which supports the results obtained previously and indicates the inhibition of fermentation at this pressure range. But, comparing these results with the initial phase of fermentation at 100 MPa it is verified that, in the latter case, the sugars concentration decreases, mainly in the initial phase of fermentation, which can indicate that bacteria is active and should need energy to perform adaptation mechanisms to withstand pressure stress. So, the obtained results for this parameter, during pre-treatment, can be derived by an experimental error in analysis performance and/or presence of interfering substances in the sample.

After the sample transference to a bath at atmospheric pressure, it is verified that the sugars concentration decreases during the fermentation time. At the first 90 minutes of fermentation, is possible to note that there was no decrease in sugars concentration, which can be explained by microbial adaptation to the new growth conditions. But, this is not in accordance with the results for product formation previously described (Figure 21 and 22), where after pre-treatment there was no adaptation time for the pressure conditions change, so this results can be derived once again from an experimental error.

Furthermore, after approximately 360 minutes of fermentation at atmospheric pressure, the reducing sugars concentration stabilize at 44.00 g/L. Samples without pre-treatment has the a similar profile for this parameter, stabilizing the concentration at around 41.00 g/L, thus the final concentration of sugars is slightly higher for samples with pre-treatment ($p < 0.05$).

In conclusion, the increase of 100 MPa pre-treatment's time influences negatively the rate of the subsequent fermentation rate at atmospheric pressure, which can be explained by cell death and/or irreversible inhibition when the samples are under HHP. Despite that, it is verified that at 100 MPa there was also a reversible inhibition of bacteria because when the pressure conditions changed, they are capable to ferment and produce yogurt, however this occurs slowly.

1.3. Monitoring Specific Physicochemical Parameters during Yogurt's Production

In order to complement the study of pressure effects in lactic acid fermentation of yogurt, it was performed different analyses with the purpose of monitor others physicochemical parameters, that are interesting to supplement the information previously obtained. For that, some pressures with interesting results was chosen, i.e. 5 MPa for having a more similar profile to atmospheric pressure, 100 MPa because fermentation does not occurs at this pressure range but others compounds can be produced; and fermentation with combined pressure conditions to verify if there were changes in parameters that were not measure yet. Fermentation at atmospheric pressure (0.1 MPa) was used as control.

In addition, it is important to note that, before performing the analyses from this section, the samples were centrifuged and only the supernatant was analyzed, as described in Material and Methods section. So, the present methods are only able to analyze the compound concentration in extracellular medium and not in the intracellular medium.

For these results, a statistical analysis was also carry out with the purpose of verify if differences between the analyzed samples are significant or not. The results obtained for this analysis are represented in Appendix II – section c).

1.3.1. D-Glucose Concentration

One of the analyzed parameters is D-Glucose concentration and the obtained results for the analyzed fermentations are presented in Figure 24.

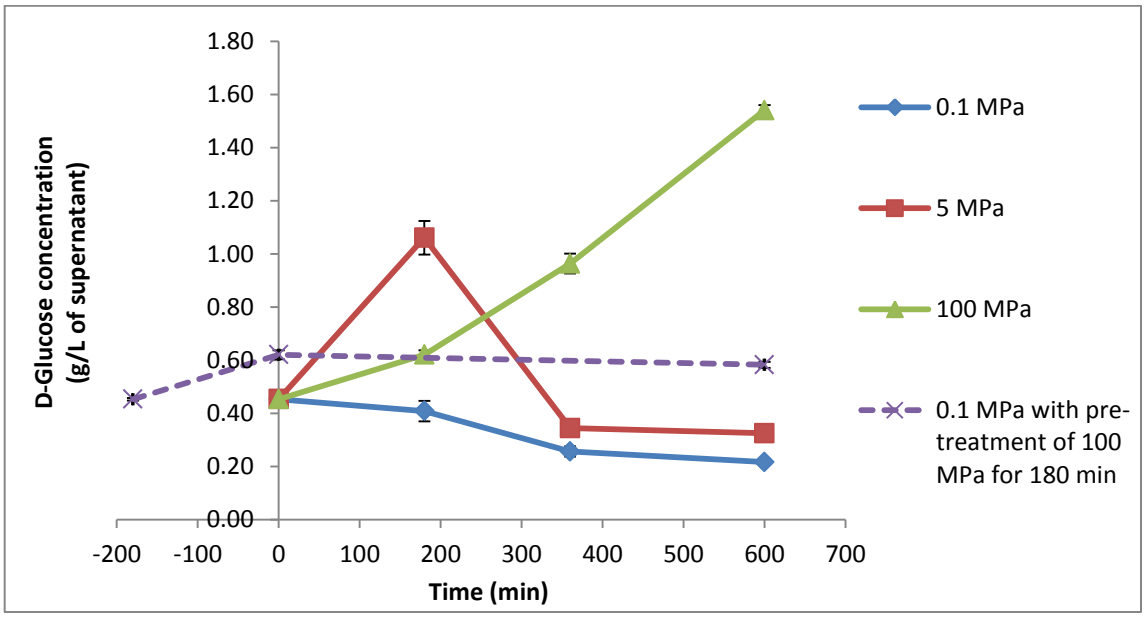

Figure 24. D-Glucose concentration during fermentation time, under 5MPa, 100 MPa and combined pressure conditions (pre-treatment of 100 MPa for 180 minutes). Fermentation at atmospheric pressure was used as control.

During fermentation at 0.1 MPa, there was a decrease on extracellular D-glucose concentration (from 0.45 g/L to 0.22 g/L), but it is possible to note that this sugar is present in low levels in extracellular medium. This is explained by the fact that in milk, i.e. in the beginning of fermentation, the main sugar is lactose that is hydrolyzed in D-glucose and D-galactose by starter cultures and the former one is consumed by them. Therefore, during fermentation time, some of the remaining D-glucose present in milk is consumed by yogurt's bacteria, in addition to D-glucose derived from lactose (parameter not evaluated).

Regarding to fermentation under 5 MPa, it is possible to note that, in the beginning, there is an increase of extracellular D-glucose concentration, which can perhaps be explained by a microbial excessive hydrolysis of lactose present in milk. This can occur due to an activity increase of β-galactosidase (enzyme responsible for lactose hydrolysis) under pressure, which leads to a higher D-glucose production. But, as the utilized method

only analyze the compound concentration in extracellular medium and considering that the hypothesis presented above is correct, the increase showed in Figure 24 can be derived from D-glucose expelling to medium, as they perform with D-galactose moiety [6]. This D-glucose expelling can be due to a marked increase of D-glucose concentration in cells because of the activity increase of β-galactosidase, and, as the fermentative process are inhibited with pressure, they may not consume all D-glucose moiety present in intracellular medium, so the cells starts to expel it to extracellular medium. But after the pressure adaptation is complete, the fermentative process occurs at a higher rate than in the beginning of fermentation and the D-glucose that was expelled to extracellular medium is consumed, since the D-glucose concentration decrease (from 1.06 g/L to 0.34 g/L). In the final phase of fermentation, D-glucose concentration stabilize, which can indicate that the D-glucose consumed by cells come from lactose hydrolysis as usually. Other explanation for that D-glucose concentration increase verified in fermentation at 5 MPa, can be an experimental error in analysis realization and/or problems with sample.

Comparing fermentation at 5 MPa to control one (0.1 MPa), it is verified that despite of 180 minutes' sample, the profile of D-glucose concentration is similar until 360 minutes of fermentation ($p > 0.05$), but at the end of fermentation (600 minutes), the D-glucose concentration is significantly higher for fermentation at 5 MPa ($p < 0.05$), as verified in reducing sugars concentration (Figure 11).

In Figure 24, it is also represented the D-glucose concentration during fermentation under 100 MPa and it is verified that there is an increase over all analyzed time (from 0.45 to 1.54 g/L). This can be once again explained by increase of β-galactosidase activity while the fermentative process is inhibited, in this case the process is completely inhibited since there was not lactic acid production in fermentation under this pressure range (Figure 9). Thus, there was an increase of D-glucose concentration in intracellular medium which leads to D-glucose expulsion to extracellular medium, as occurs in the beginning of fermentation at 5 MPa.

During analysis to reducing sugars concentration (Figure 11), this concentration remains slightly constant during fermentation time at 100 MPa and the increase of D-glucose concentration showed in Figure 24 is not verified. This can be explained by the difference in values range of each analysis, since the reducing sugars concentration, which corresponds to lactose, glucose and galactose concentration, is significantly higher than D-

glucose concentration. Thus, the obtained values in this section, namely this concentration increase, are hidden in reducing sugars concentration.

Furthermore, it is also analyzed the D-glucose concentration of samples with 100 MPa pre-treatment for 180 minutes. So, analyzing Figure 24, it is verified that during the pre-treatment there is an increase in D-glucose concentration (from 0.45 g/L to 0.62 g/L) as occurs in fermentation under 100 MPa. But when the samples are transferred to a bath at atmospheric pressure, the concentration remains apparently constant during fermentation, despite the fact that the fermentation occurs over this time, as verified above (Figure 21 and 22). In this case, it is important to note that the fermentation monitoring of these samples over the 600 minutes that they are at atmospheric pressure is impossible, since it was only analyzed samples correspondents of 0 and 600 minutes of atmospheric pressure fermentation. Nevertheless, the obtained results may indicate that lactose hydrolysis occurs at the same rate than fermentation, i.e. D-glucose consumption and lactic acid formation by starter cultures occurs simultaneously, because D-glucose concentration remains constant over the time of fermentation at atmospheric pressure. In addition, it is verified that the final D-glucose concentration value is significantly higher ($p < 0.05$) than the obtained for the fermentations at 5 MPa and atmospheric pressure, which may indicate that the extracellular D-glucose is not consumed by starter cultures in this fermentation.

To verify if these changes in D-glucose concentration are possible, i.e. D-glucose concentration increase, the obtained D-glucose concentration, if all lactose present in milk were hydrolyzed into its monomers, was calculated. By these calculations, which are explained in Appendix IV, it was verified that the maximum of D-glucose concentration obtained in this analysis, i.e. 1.54 g/L after 600 minutes at 100 MPa, corresponds only to ≈ 5 % of the estimated value for D-glucose obtained if all lactose were hydrolyzed. Thus, the D-glucose amount that was expelled during fermentation at 100 MPa is much lower than the total amount that could be expelled if all milk lactose were hydrolyzed. Nevertheless, the results clearly show differences when fermentation occurs under pressure or when a pressure pre-treatment is applied.

1.3.2. L-/D-Lactic Acid Concentration

Others parameters evaluated in this section were L- and D-Lactic acid concentration and the obtained results for fermentations analyzed are presented in Figure 25 and 26, respectively.

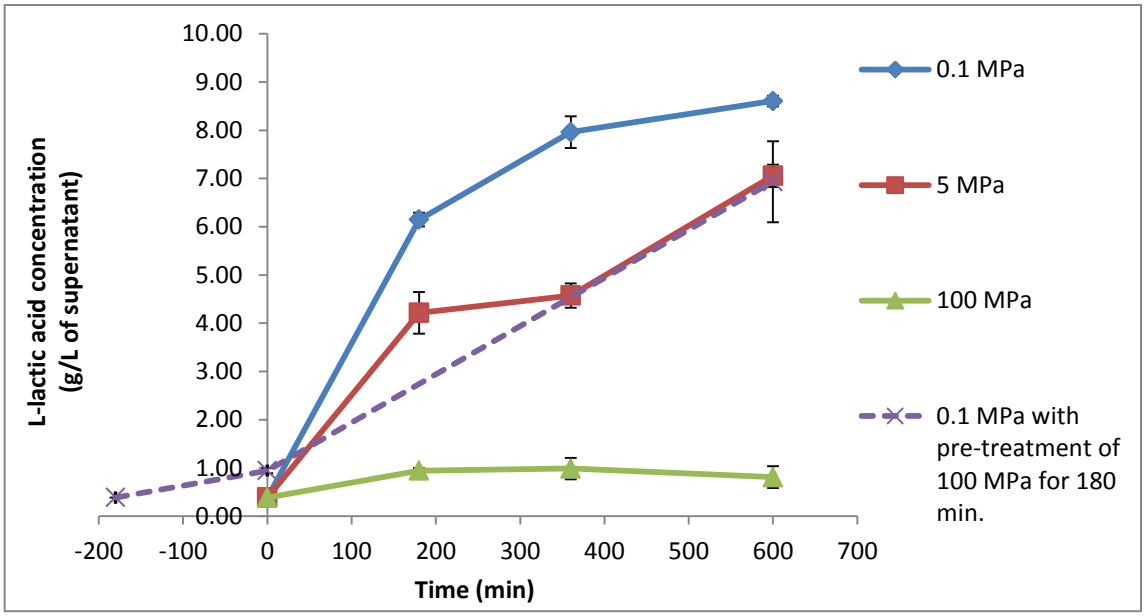

Figure 25. L-Lactic acid concentration during fermentation time, under 5MPa, 100 MPa and combined pressure conditions (pre-treatment of 100 MPa for 180 minutes). Fermentation at atmospheric pressure was used as control.

In fermentation at atmospheric pressure, as expected, the L-lactic acid concentration increased during fermentation time (from 0.39 to 8.61 g/L), but in the end of fermentation the production rate decrease, as it was previously verified in Figure 9. This is explained by the fact that the growth of microorganism responsible for production of L-lactic acid, namely *S. thermophilus* [37, 38], is inhibited by the increase of acidity of extracellular medium [1], i.e. there is product inhibition in this case.

Regarding to fermentation at 5 MPa, it is possible to note that in the beginning of fermentation, there is a significant increase of L-lactic acid concentration (from 0.39 to 4.21 g/L), but between the 180 and 360 minutes of fermentation, the concentration stabilize. Despite that, in the end of fermentation, L-lactic acid concentration increases again (from 4.57 to 7.06 g/L), reaching a final concentration significantly similar to

fermentation at atmospheric pressure ($p > 0.05$). These results are in accordance to those obtained in titratable acidity analysis (Figure 9) since there is production of L-lactic acid under these conditions but in a lower rate than at atmospheric pressure, but in the end the values for both fermentations are similar. Therefore, it is possible to conclude that at this pressure there is some inhibition of *S. thermophilus* activity, when compared to its activity at atmospheric pressure.

Furthermore, the determination of L-lactic acid concentration for fermentation at 100 MPa was performed and it was verified that there was no variation in L-lactic concentration over the fermentation time. These results are in accordance to the results obtained previously to titratable acidity (Figure 9) and point out once again that at this pressure, the fermentation does not occur, which can be due to inhibition of cells metabolism, namely of *S. thermophilus*, or even its death.

In addition, the L-lactic acid concentration during fermentation at atmospheric pressure with a pre-treatment of 100 MPa for 180 minutes was also measure. As mentioned previously, during fermentation at 100 MPa there was no fermentation, which is reflected in L-lactic acid production, i.e. there was no acid production. Therefore, as expected, this is also verified during pre-treatment of 100 MPa, since there was no production of this isomer. But, when samples were transferred to a bath at atmospheric pressure, there was a marked increase of L-lactic acid production rate, so it can be point out that S. *thermophilus* cells were only reversible inhibited during pre-treatment and when the pressure conditions change they overcome the stress and start the fermentation. These results are in accordance with the ones obtained previously to titratable acidity (Figure 21). In addition, it is possible to note that, in the end of the 600 minutes at atmospheric pressure, the L-lactic acid concentration is similar to fermentation at 5 MPa (6.93 g/L and 7.06 g/L, respectively, $p > 0.05$), which may indicate that the cells recover the activity to one similar to the cells at 5 MPa. As there are no points during the fermentation at atmospheric pressure after pre-treatment, it is impossible to infer the complete profile of L-lactic acid production.

In addition to L-lactic acid, D-lactic acid concentration was also analyzed, since during fermentation both isomers of lactic acid are produced [6], since it is known that each starter culture produces mainly one isomer, *S. thermophilus* the L-lactic acid and *L.*

bulgaricus the D-lactic acid [37-39]. For the latter one, the obtained results for analyzed fermentations are represented in Figure 26.

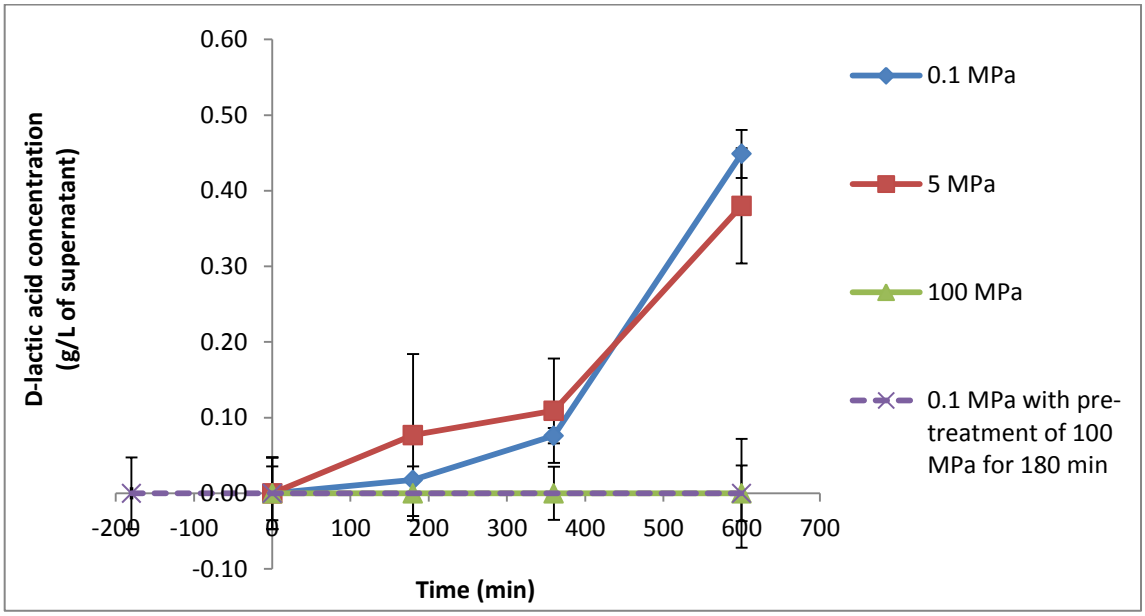

Figure 26. D-Lactic acid concentration during fermentation time, under 5MPa, 100 MPa and combined pressure conditions (pre-treatment of 100 MPa for 180 minutes). Fermentation at atmospheric pressure was used as control.

Note: Values represented as 0.00 g/L correspond to samples where it was impossible to quantify D-lactic acid by the analysis' method employed.

Analyzing Figure 26, it is possible to note that D-lactic acid concentrations are much lower than L-lactic acid ones. This difference can be due to amount of two starter cultures in fermentative medium that, generally in industrial processes, is higher for *S. thermophilus* than for *L. bulgaricus* [6], and the inoculum used in this work is a commercial yogurt.

Regarding to fermentation at atmospheric pressure, it is verified that in the beginning of fermentation (first 360 minutes), the rate of D-lactic acid production is low, but have a marked increase in final phase (from 0.08 g/l to 0.45 g/L). Taking into account that the bacteria responsible for D-lactic acid production, namely *L. bulgaricus*, is only able to produce D-lactic acid in an advanced stage of fermentation, in contrast to *S. thermophilus*, that are capable to produce L-lactic acid in the beginning [36], this profile is

in accordance to literature. Hence, there is no such higher D-lactic acid production in the initial phase of fermentation, as L-lactic acid production (Figure 25).

In fermentation under 5 MPa, it is verified that, in contrast to fermentation at atmospheric pressure, the D-lactic acid production starts in the initial stage of fermentation in a higher rate, reaching 0.11 g/L after 360 minutes. These results can indicate that under pressure there was some metabolism change of *L. bulgaricus*, since the D-lactic acid production profile is different of the one correspondent to fermentation at atmospheric pressure. Despite of this, in the final phase of fermentation there was also a marked increase of D-lactic acid concentration (until 0.38 g/L), where the final value is similar to the final concentration in fermentation at atmospheric pressure ($p > 0.05$). Therefore, although the D-lactic acid production profile is slightly different between these two pressures conditions, the concentration at the end of fermentation is similar, which can indicate that *L. bulgaricus* yield is not significantly affected when the fermentation takes place under these ranges of pressure.

For fermentation under 100 MPa, the D-lactic acid concentration remains below the quantification range for the analysis method employed, so it was considered that the concentration was 0.00 g/L. This can indicate that at this pressure the *L. bulgaricus* is incapable to ferment and produce D-lactic acid, due to cell inhibition and/or death, which is in accordance to literature that shown that this bacteria is more sensible to pressure than *S. thermophilus* [144, 145].

Regarding to samples that were pre-treated during 180 minutes before the fermentation at atmospheric pressure, this profile is also verified, but not only during pre-treatment but also during fermentation itself. This can be explained by irreversible inhibition of *L. bulgaricus*, during pre-treatment, that after that they are unable to overcome, even if they are at optimal growth conditions, or it can also be due to cell death during pre-treatment.

To complement these analyses and to relate the concentration of these two isomers during fermentation time, the ratios between the concentrations of L- and D-lactic acid was calculated, called L-:D-lactic acid ratio, and the results are represented at Appendix V. These ratios was only calculated to fermentations at atmospheric pressure and 5 MPa, since with the others pressure conditions analyzed were not verified D-lactic acid production to

compare with L-lactic acid concentration, which makes impossible to calculate L-:D-lactic acid ratio. In fermentation at atmospheric pressure, it is possible to point out that, in the beginning, the L-:D-lactic acid ratio is high (≈ 350), decreasing during the fermentative process. While that, in fermentation under 5 MPa, the L-:D-lactic acid ratio also decrease during fermentation time, but not in a markedly way as fermentation in atmospheric pressure. This is supported by results obtained previously, since in the beginning of fermentation at atmospheric pressure, is produced a great amount of L-lactic acid but little of D-lactic acid but, under 5 MPa, D-lactic acid production starts at the very beginning. In addition, it is also possible to note that in the end of fermentation (600 minutes), the L-:D-lactic acid ratio of these two pressure conditions is similar. So, the results for lactic acid ratios indicate that D-lactic acid production increases relatively to L-lactic acid production during fermentation time in both cases, and, furthermore, in the end the proportion of each isomer is similar, which means that for both analyzed samples the L-lactic acid concentration is approximately 19-fold D-lactic acid concentration. Thus, despite the fermentation at these two pressure conditions has different product profile and at 5 MPa there was a decrease in acid production, in the end of these two fermentations (600 minutes), the relative amount for each lactic acid isomer produced is similar, which can indicate that the starter cultures have the same behavior regarding to acid production.

In conclusion, the obtained results for production of both lactic acid isomers during fermentation and their proportion are in accordance to literature because usually the industrial starter cultures are constituted by a higher amount of *S. thermophilus* and a lower of *L. bulgaricus* [6]. The main reason for this proportion of industrial starters is that D-lactic acid is produced by *L. bulgaricus* and this isomer, when compared to L-lactic acid produced by *S. thermophilus*, is metabolized slower in man and if consumed in excess, causes metabolic disorders [6].

1.3.3. Acetaldehyde Concentration

In addition, it was analyzed the acetaldehyde concentration of samples and the obtained results for different pressure conditions used in this section are presented in Figure 27. This compound is one product of the lactic acid fermentation of yogurt and it is

considered as the major responsible for yogurt's flavor [58, 61, 62], thus this parameter was analyzed.

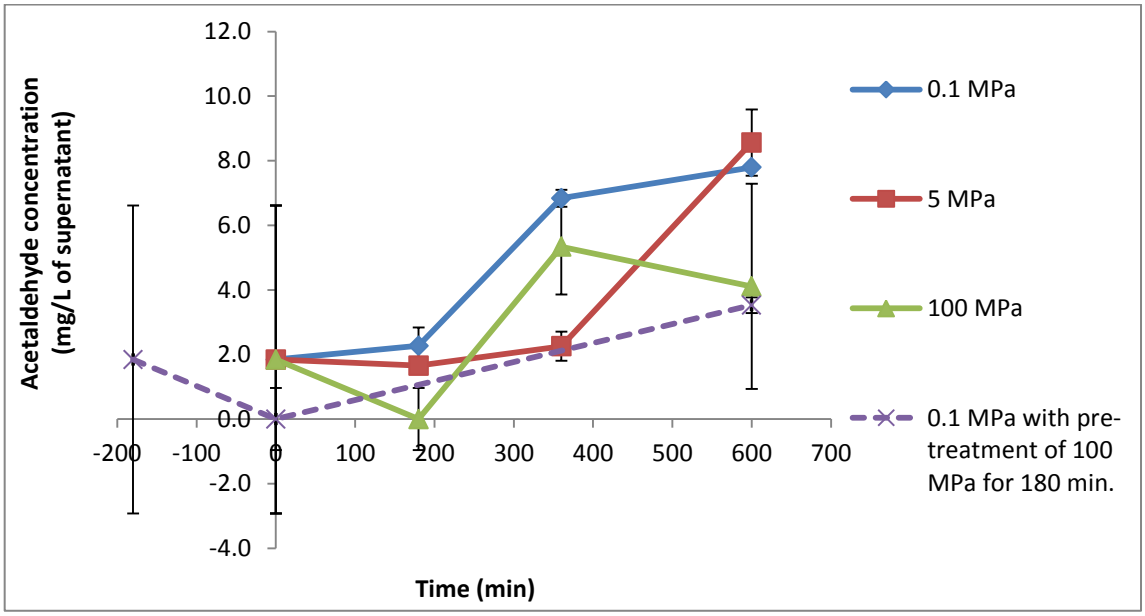

Figure 27. Acetaldehyde concentration during fermentation time, under 5MPa, 100 MPa and combined pressure conditions (pre-treatment of 100 MPa for 180 minutes). Fermentation at atmospheric pressure was used as control.

Note: Values represented as 0.0 mg/L correspond to samples where it was impossible to quantify acetaldehyde by the analysis' method employed.

In Figure 27, it is possible to note that the calculated concentrations for this parameter are very low, in range of mg/L, which is in accordance to the literature, since usually the acetaldehyde concentration for commercial yogurts obtained from mixed cultures are between 2 mg/L and 42 mg/L [152]. Therefore, the obtained values are in the concentration range described in literature.

For fermentation at atmospheric pressure, it is possible to note that there was acetaldehyde production during fermentation time, with a higher production rate between 180 and 360 minutes (from 2.3 to 6.8 mg/L). In the beginning, there was no concentration increase, which can be due to an adaptation phase of bacteria and their metabolic pathways, for production of this compound. After the 360 minutes of fermentation, the acetaldehyde production slows down and reaches a final value of 7.8 mg/L, which are in accordance to literature, namely the acetaldehyde concentration in yogurt.

In pressurized samples at 5 MPa, it is verified that the main production of this compound occurs in the final phase of fermentation (between 360 and 600 minutes) and until that time the concentration slightly increase (from 1.8 to 2.3 mg/L). So, the acetaldehyde's production profile for fermentation at 5 MPa are different of the one for fermentation at atmospheric pressure, which can be due to the need of a longer adaptation time when cells are subjected to pressure. In addition, in the end of fermentation, it is verified that the final value of acetaldehyde concentration is similar to the final value for fermentation at atmospheric pressure (8.6 mg/L and 7.8 mg/L, respectively, $p > 0.05$). These results indicate that the final product of both these fermentations has a similar flavor, due to importance of acetaldehyde for yogurt's flavor. In order to compare the final products obtained with these two fermentations (0.1 and 5 MPa), an informal sensorial analysis with the laboratorial staff (6 persons) was performed, where was evaluated the yogurt's flavor. In this analysis, it was verified that both yogurts has a similar flavor, which are in accordance with the obtained results for acetaldehyde concentration.

For fermentation under 100 MPa, in the initial phase, there is a decrease in acetaldehyde concentration to values that the analysis method is unable to quantify. This decrease can be explained by degradation of the some acetaldehyde that may be present in milk. After that, there is an increase in acetaldehyde concentration, so despite the fact that starter cultures do not ferment, i.e. there was no lactic acid production, at this pressure condition, there was acetaldehyde production, which can explain the sugars consumption previously observed (Figure 11). But in the end of fermentation, despite the acetaldehyde concentration slightly decreases, which can be interpreted as concentration stabilization, this value is significantly similar to the final concentrations of fermentations represented ($p > 0.05$). So, it can be concluded that, after 180 minutes of fermentation, bacteria are able to produce acetaldehyde but this production stops after 360 minutes of fermentation, which can be explained by decrease and/or inhibition of bacteria activity. But it is important to note that the error associated to samples fermented at this pressure is larger than to the samples fermented at others pressure conditions, so the profile described for acetaldehyde production cannot be the real one.

In Figure 27 are also represented the results for samples that were fermented under combined pressure conditions. By analysis of the obtained results for these samples, it is verified that, during pre-treatment, there is a decrease in acetaldehyde concentration, as in

the initial phase of fermentation under 100 MPa, described previously. But, when the samples were transferred to perform the remaining fermentation time at atmospheric pressure, it is verified that there was acetaldehyde production, reaching a final value of 3.5 mg/L. Thus, although the final value is smaller than the ones for others tested fermentations, these values are significantly similar ($p > 0.05$). Therefore, there is production of acetaldehyde during fermentation at atmospheric pressure after the pre-treatment (100 MPa for 180 minutes). So, despite the fact that in the end of this fermentation it is obtained yogurt as final product (pH necessary is reached – Figure 22), the flavor is not similar to a yogurt obtained from fermentation at atmospheric pressure, because the final acetaldehyde concentration is slightly different.

1.3.4. Ethanol Concentration

Furthermore, an analysis of ethanol concentration to the obtained samples was also performed, in order to verify if the fermentation conditions tested, namely under pressure and combination of pressure conditions, change the fermentative bacteria's metabolism and they became heterofermentative (production of CO_2 and ethanol, in addition to lactic acid). This hypothesis was considered, since some samples presented a swollen aspect, which samples fermented at atmospheric pressure did not present. It was suggested that this samples appearance may be due to CO_2 production and thus the metabolism change hypothesis emerged.

Regarding to the obtained results for this analysis, these are not represented here because all of them are below the quantification limit imposed by the method used. Thus, it is possible to conclude that there was no significant ethanol production during the tested fermentations, which indicates that there was no metabolism change as hypothesized previously.

2. MICROBIOLOGICAL ANALYSIS OF YOGURT'S FERMENTATIVE BACTERIA

In order to evaluate the starter cultures viability, a microbiological analysis to yogurt fermentative bacteria, i.e. *S. thermophilus* and *L. bulgaricus* was performed, during

the fermentative time, under different pressure conditions (same conditions used in the last section). Furthermore, a statistical analysis was also carry out for the obtained results in this section, which has as main purpose check if differences between the analyzed samples are significant or not and the obtained results for that are present in Appendix II – section d).

The obtained results for each microorganism viability are represented in Figure 28 and 29 for *S. thermophilus* and *L. bulgaricus*, respectively.

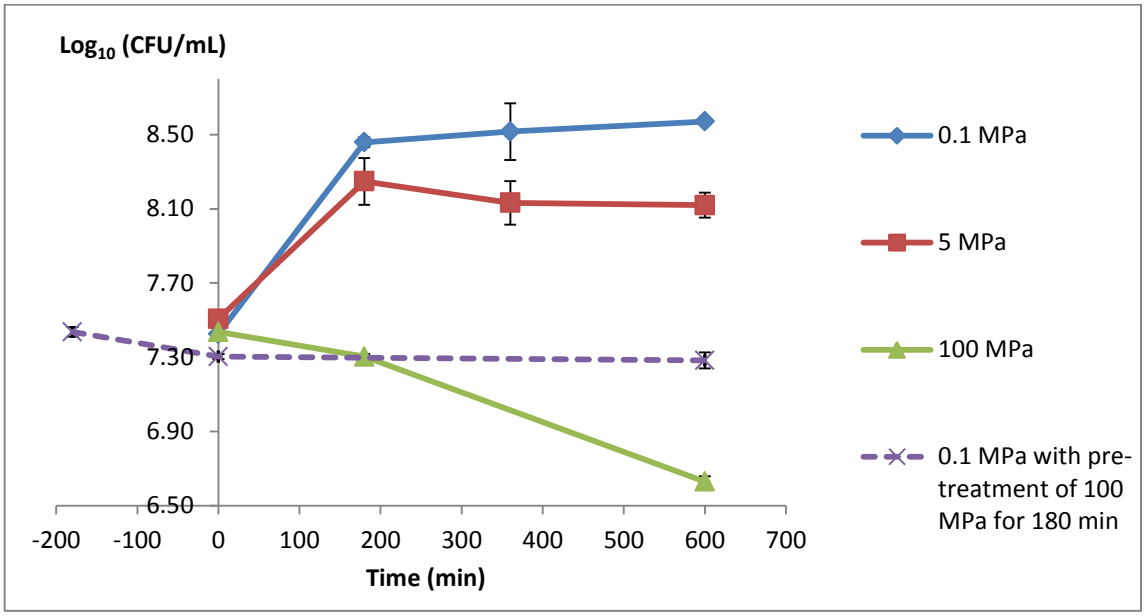

Figure 28. *Streptococcus thermophilus* count during fermentation time, under 5MPa, 100 MPa and combined pressure conditions (pre-treatment of 100 MPa for 180 minutes). Fermentation at atmospheric pressure was used as control.

Regarding to fermentation at atmospheric pressure, it is verified that the microbial load of *S. thermophilus* has a marked increase in the initial phase of fermentation (from 7.44 log CFU/mL to 8.46 log CFU/mL) and after that stabilize around 8.50 log CFU/mL. The obtained results for this pressure are in accordance with literature, since the growth of this microorganism is inhibited in the end of lactic acid fermentation due to the increase of acidity of extracellular medium [1]. Furthermore, as previously mentioned, this microorganism are responsible for production of L-lactic acid [37, 38], so these results can be compared with the results obtained for production of this isomer, which are represented

in Figure 25. So, analyzing both results, it is verified that they are in accordance with each other, since the profile of L-lactic acid production is similar to the profile of *S. thermophilus* growth.

For fermentation under 5 MPa, it is verified that the growth profile of *S. thermophilus* is similar to the obtained for samples non-pressurized. In the beginning of fermentation, there was also a marked increase of *S. thermophilus* load, reaching the value 8.25 log CFU/mL, which is similar to the correspondent load value of fermentation at atmospheric pressure (8.46 log CFU/mL, $p > 0.05$). This difference between the two pressure conditions is also verified in L-lactic acid concentration (Figure 25). In an advanced phase of fermentation, it is verified a decrease in this microorganism load (8.25 to 8.13 log CFU/mL) followed by stabilization around 8.13 log CFU/mL. This can indicate that after 180 minutes of fermentation, *S. thermophilus* growth is in stationary phase, which is not accompanied by L-lactic acid production that increase in the end of fermentation (Figure 25). This difference between the profiles of bacterial growth and product formation can mean that *S. thermophilus* growth is not directly related with L-lactic acid production, which can be compared to the Crabtree effect described by several yeasts species, as *Saccharomyces cerevisiae*. The Crabtree effect is explained by biomass production without fermentation in some conditions and by fermentation without microorganisms growth in others conditions [159].

During fermentation at 100 MPa, it was verified that *S. thermophilus* load decreases approximately 1 log unit. These results are in accordance with results previously obtained for acid production, where it was concluded that there was no fermentation at 100 MPa. In addition, it is important to point out that despite the microbial load decrease during fermentation time, at the end of fermentation, the *S. thermophilus* load is still high (6.63 log CFU/mL). Therefore, although some bacteria are destroyed by pressure, there are bacteria that are able to overcome this stress and survive, thus their viability is not totally lost. But, despite this, there was no L-lactic acid production (Figure 25), so at these conditions *S. thermophilus* present in the medium may not perform lactic acid fermentation.

Regarding to samples with a pre-treatment of 100 MPa for 180 minutes, it is possible to verify that during pre-treatment, there was a slightly decrease in *S. thermophilus* load (from 7.44 to 7.30 log CFU/mL), as observed in the beginning of fermentation under 100 MPa. In addition, these results are in accordance with the results of

L-lactic acid production correspondent, since during pre-treatment there was no acid production. When the samples are transferred for a bath at atmospheric pressure, the *S. thermophilus* load remains constant (at ≈ 7.30 log CFU/mL) during 600 minutes of fermentation. This profile is not followed by L-lactic acid production, since after pre-treatment there was a significant increase in this acid production. Thus, it is possible to point out that, in this phase, is occurring once again a similar effect to Crabtree effect for yeasts, as described previously in fermentation at 5 MPa [159].

In conclusion, it was observed an effect similar to Crabtree effect on *S. thermophilus* growth and L-lactic acid production. This effect appears to be triggered when samples are subject to pressure, since it was observed in an advanced stage of fermentation at 5 MPa and when the samples were transferred to atmospheric pressure after pre-treatment with 100 MPa. Therefore, it is possible to point out that the effect of pressure in *S. thermophilus* is the fact that is not necessary that bacteria grow to produce L-lactic acid and vice-versa.

As previously stated, it was also evaluated the *L. bulgaricus* growth and consequently its viability during the fermentative processes tested and the obtained results are represented in Figure 29.

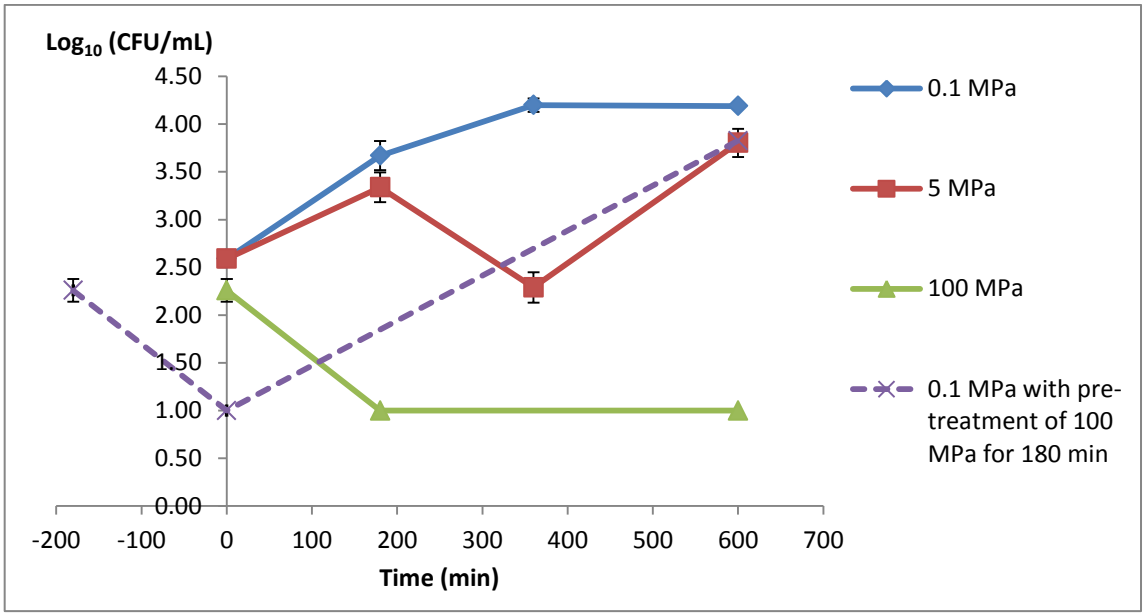

Figure 29. *Lactobacillus bulgaricus* count during fermentation time, under 5MPa, 100 MPa and combined pressure conditions (pre-treatment of 100 MPa for 180 minutes). Fermentation at atmospheric pressure was used as control.

Note: Values of log(CFU.g^{-1}) = 1 correspond to samples with log(CFU.g^{-1}) ≤ 1 (count below the detection level).

For control fermentation (at atmospheric pressure), it was verified that there was an increase of *L. bulgaricus* load (from 2.59 to 4.20 log CFU/mL), but after 360 minutes of fermentation, this microbial load stabilize around 4.20 log CFU/mL, which may indicate that cultures are in stationary phase. Comparing this results to the respective D-lactic acid production (Figure 26), is possible to verify that despite the load increased in an initial phase of fermentation, the D-lactic acid concentration is stable in the beginning, only increasing after 360 minutes of fermentation. Therefore, it is possible to points out that the *L. bulgaricus* growth and D-lactic acid production are not directly related with each other, which can be explained as previously for *S. thermophilus* and its L-lactic acid production. In addition, comparing the profile of both starters' growth, it is possible to verify that the increase of *L. bulgaricus* load during fermentation time (≈ 1.5 log units) is higher than the respective increase of *S. thermophilus* load (≈ 1.0 log unit). But the amount of *S. thermophilus* is higher than *L. bulgaricus* amount during all fermentative process, which is in accordance with the results obtained for concentration of the two lactic acid isomers

(higher to L-lactic acid) and with literature - *L. bulgaricus* is present in lower amount than *S. thermophilus* in yogurt [6].

In addition, it is possible to note that fermentations under pressure have a different profile when compared to fermentation at atmospheric pressure. At 5 MPa, there was an increase in *L. bulgaricus* load in the first 180 minutes of fermentation (from 2.59 to 3.34 log CFU/mL), reaching a microbial load slightly lower than fermentation at atmospheric pressure (3.67 log CFU/mL), but no significantly different ($p > 0.05$). This indicates that, at these range of pressures, *L. bulgaricus* are able to overcome the stress and growth. But, in an intermediate phase of fermentation, there was a decrease of microbial load until values slightly lower than initial values (2.29 log CFU/mL and 2.59 log CFU/mL, respectively). This indicates that during this time the cells were destroyed, which can be explain by production of a prejudicial compound to *L. bulgaricus* growth and development, causing its death. Furthermore, in the final stage of fermentation, there was a significant increase in *L.bulgaricus* load reaching a final value of 3.83 log CFU/mL. This can be explained by death of cells sensible to the produced compound in the intermediate stage and then the resistant ones are able to develop and growth in a higher rate. The obtained profile for *L. bulgaricus* viability during fermentation under 5 MPa is reflected in D-lactic acid concentration (Figure 26) because in intermediate phase of fermentation where there is a decrease of microbial load, there is a stabilization of acid concentration and when there is growth of *L. bulgaricus*, there is production of D-lactic acid.

But, other explanation for the decrease of *L. bulgaricus* load and stabilization of D-lactic acid concentration during fermentation at 5 MPa can be an experimental error associated to sample and/or quantification analyses, since the results for 360 minutes' sample, in both cases, are no consistent with the others obtained results.

In fermentation under 100 MPa, it is possible to verify that *L. bulgaricus* load decrease steeply and, after 180 minutes, the bacteria count is already below the quantification level of the performed analysis (log CFU/mL $\leq$ 1). These results are in accordance with the results obtained for all analyses performed during this work, since the results of *L. bulgaricus* count indicate that there is cells death during fermentation time and thus fermentation does not occur. In addition, the fact that this microorganism load is below quantification level at 100 MPa is in accordance to literature, since there is studies

that concluded that *L. bulgaricus* is more sensible to pressure than *S. thermophilus* after pressure treatments of 400 MPa [144, 145].

For pre-treated samples, it was verified that during pre-treatment, the *L. bulgaricus* load decrease until values below quantification level, as previously observed in the initial phase of fermentation at 100 MPa. But when the samples are transferred for a bath at atmospheric pressure, the *L. bulgaricus* load have a marked increase of approximately 3 log units, reaching values similar to final value of fermentation under 5 MPa and even of fermentation at atmospheric pressure ($p > 0.05$). These results can be explained by proliferation of cells that were able to overcome the pressure stress during pre-treatment, that despite they were below the quantification level, they were present in samples. The *L. bulgaricus* growth after pre-treatment is not in accordance with the obtained results for D-lactic acid production (Figure 26), since during the whole fermentation at atmospheric pressure after pre-treatment, D-lactic acid concentration remains below the method's quantification level. This difference in the profile between microbial growth and product formation can be explained, once again, by the effect similar to Crabtree effect, as described previously for *S. thermophilus*, because there was *L. bulgaricus* growth but there was no D-lactic acid production. In addition, the growth profile of this starter can indicate that the bacteria, which grow after pre-treatment, may have developed resistance mechanisms that can lead to production of other metabolites, which are not detectable through the analyses performed during this work.

V. CONCLUSIONS

In this work, the effect of perform yogurt's fermentation under increasing pressure conditions was studied, which it has not been studied yet. In order to monitor the performed fermentations, some physicochemical parameters and starters' viability over fermentation time was studied.

The monitoring of yogurt's fermentative process under increasing pressure conditions was performed through analyses of titratable acidity, pH variation and reducing sugars concentration, with the results indicating that pressure influences negatively the fermentation rate. Thus, with pressure increasing, there is a gradual inhibition of fermentation until stops at pressures about 100 MPa. In addition, in fermentation under 5 MPa is obtained as final product a yogurt, since the fermentation final pH corresponds to pH necessary to obtain it, but fermentation time is twice of process at atmospheric pressure.

In addition, a kinetic analysis was performed, where the Va for the three parameters described previously was calculated. It was obtained positives values for Va, which confirms the fermentation inhibition by increasing pressure. The physicochemical parameter most affected by pressure is substrate consumption, followed by pH variation and lactic acid production obtained by titratable acidity. So, the latter one is lesser affected by increasing pressure, which can indicate that, during fermentation under pressure, there is formation of different organic acids, namely with pKa lower than lactic acid.

Additionally, a fermentation under combined pressure conditions was performed, i.e. the samples were subject to a variable pressure pre-treatment and then were transferred to an atmospheric pressure medium. In all tested cases, the fermentation does not occur during pre-treatment, but, after that, the starters are able to ferment. The fermentation rate at atmospheric pressure depends of pre-treatment conditions (pressure and time). The samples with pre-treatment of 100 MPa during 90 minutes have a higher fermentation rate than samples without pre-treatment. This difference may indicate that during pre-treatment, there is pressure adaptation of cells that leads to some changes in their metabolism, which when samples are at atmospheric pressure it is reflected in an increase of fermentation rate. Meanwhile, samples with a 100 MPa pre-treatment longer (180 minutes) in the end of 600 minutes of fermentation also became yogurt, but the fermentation rate is lower than

fermentation without pre-treatment. This difference can be explained by cell death and/or irreversible inhibition, with the increase of pressure pre-treatment time. But, there is also a reversible inhibition of bacteria, since they are capable to ferment and produce yogurt, when the sample are transferred to a bath at atmospheric pressure.

In addition, others physicochemical parameters was analyzed. In D-glucose concentration analysis, it is verified that with increasing pressure there was an increase of D-glucose in extracellular medium, which can be due to milk's lactose hydrolysis in cells and subsequent expulsion of D-glucose to extracellular medium. But, when fermentation occur the D-glucose concentration decrease, since sugar is consumed by starter cultures, or remains constant because the D-glucose consumed by bacteria was derived by lactose hydrolysis and not from extracellular medium.

The concentration of both two isomers of lactic acid (L- and D-) was also calculated and the obtained results are in accordance with results obtained for titratable acidity. In addition, it was verified that L-lactic acid are more abundant in yogurt than D-lactic acid, and in the end of fermentation the proportion of both isomers is similar for fermentation at atmospheric pressure and 5 MPa.

The concentration of main compound responsible for yogurt's flavor, i.e. acetaldehyde, was also studied. During fermentation time, there was acetaldehyde production and with increasing pressure this formation process is inhibited, but not entirely, since at 100 MPa there was still acetaldehyde production. In samples with pressure pre-treatment, there was also acetaldehyde production but in lower amount than in samples without pre-treatment.

In this work, the viability of starter cultures was also studied, namely *S. thermophilus* and *L. bulgaricus*. The results for both starters are in accordance with the obtained results for physicochemical analyses, as lactic acid concentration and substrate consumption. Furthermore, it is important to point out that *S. thermophilus* is present in a higher amount than *L. bulgaricus* and it is more resistant to pressure, thus it is conclude that *S. thermophilus* has a more active role in fermentation under pressure.

VI. FUTURE PROSPECTS

In order to complete this work, in the future, it is still needed to perform several analyses for complete characterization of pressure influence on yogurt's production. So, for that, it is important to perform a sensorial analysis to the samples obtained by pressure treatments and, furthermore, an analysis to its rheological parameters and its microstructure. These analyses can point out the differences in the three levels analyzed between the pressure fermented samples with yogurts fermented at atmospheric pressure.

In addition, an analysis to the activity of microorganisms involved in lactic acid fermentation of yogurt is also interesting to be analyzed in a future work, since the starters have several health benefits as described previously. So, this analysis can indicate if lactic acid fermentation under pressure has negative or positive effects to microbial activity. In conclusion, in the future, is important to perform these analyses to more fully understand the final effects of pressure applied to yogurt production, both in final product and starters' cultures.

Furthermore, it can be performed a different experiment where the temperature of fermentative process under pressure is changed, in order to verify which pressure/temperature set is optimal for yogurt's fermentative process rate.

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APPENDICES

I. DNS Reagent Preparation

II. Statistical Analysis

III. Kinetics Analysis
   a. pH
   b. Titratable Acidity
   c. Reducing Sugars

IV. Estimation of Maximal Glucose Concentration in Yogurt

V. Determination of L-:D-Lactic Acid Ratios

Appendix I:

DNS Reagent Preparation

10 g of DNS were weighted and dissolved in 200 mL of a 2N NaOH solution. The solution was then heated and stirred intensively. Simultaneously a solution of 300 g of potassium tartrate in 500 mL of distilled water was prepared and heated (with intense stirring). Both solutions were mixed and stirred. Distilled water was added to make up 1 L.

Appendix II:

Statistical Analysis

A statistical analysis was carried out to different sections of this work and the obtained results are presented above. So, the significant differences ($p < 0.05$) between samples, for the same time of fermentation, are represented by different letters. When the table cells are filled with grey, it was not possible to perform this statistical analysis because the required conditions to that are not satisfied.

a) Monitoring Yogurt's Production under Pressure

Table 1. Statistical analysis performed for titratable acidity.

		Time (minutes)							
		0	**90**	**165**	**210**	**240**	**360**	**480**	**600**
Pressure (MPa)	**0.1**	*b*	*a*	*a*		*a*	*a*	*a*	*a*
	5	*b*	*b*	*b*		*b*	*bc*		*a*
	15	*b*	*bc*	*b*		*b*	*b*		
	30	*b*	*bc*		*a*		*b*	*b*	
	50	*b*	*c*		*a*		*bc*		*b*
	100	*a*	*c*		*a*		*c*		*c*

Table 2. Statistical analysis performed for pH variation.

		Tempo (minutes)							
		0	**90**	**165**	**210**	**240**	**360**	**480**	**600**
Pressão (MPa)	**0.1**	*ab*	*d*	*c*		*c*	*e*	*b*	*d*
	5	*b*	*c*	*b*		*b*	*d*		*c*
	15	*b*	*c*	*a*		*a*	*d*		
	30	*b*	*b*		*c*		*c*	*a*	
	50	*b*	*ab*		*b*		*b*		*b*
	100	*a*	*a*		*a*		*a*		*a*

Table 3. Statistical analysis performed for reducing sugars concentration.

		Tempo (minutes)							
		0	**90**	**165**	**210**	**240**	**360**	**480**	**600**
Pressão (MPa)	**0.1**	*a*	*a*	*b*		*c*	*d*	*b*	*c*
	5	*a*	*a*	*a*		*b*	*cd*		*b*
	15	*a*	*a*	*a*		*a*	*bc*		
	30	*a*	*a*		*b*		*b*	*a*	
	50	*a*	*a*		*a*		*a*		*a*
	100	*a*	*a*		*b*		*a*		*a*

b) Monitoring of Yogurt's Production under Combined Pressure Conditions

Table 4. Statistical analysis performed for titratable acidity. (*Pre-treatment of 100 MPa for 90 minutes)

		Time (minutes)				
		0	**90**	**210**	**360**	**600**
Pressure (MPa)	**0.1**	*b*	*a*		*b*	*b*
	100	*a*	*c*	*b*	*c*	*c*
	0.1 (Pre-treated)*	*b*	*b*	*a*	*a*	*a*

Table 5. Statistical analysis performed for pH variation. (*Pre-treatment of 100 MPa for 90 minutes)

		Time (minutes)				
		0	**90**	**210**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*c*		*b*	*b*
	100	*a*	*a*	*a*	*a*	*a*
	0.1 (Pre-treated)*	*a*	*b*	*b*	*c*	*b*

Table 6. Statistical analysis performed for reducing sugars concentration. (*Pre-treatment of 100 MPa for 90 minutes)

		Time (minutes)				
		0	**90**	**210**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*b*		*c*	*c*
	100	*a*	*a*	*a*	*a*	*a*
	0.1 (Pre-treated)*	*b*	*ab*	*b*	*b*	*b*

Table 7. Statistical analysis performed for titratable acidity. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)					
		0	**90**	**210**	**360**	**510**	**600**
Pressure (MPa)	**0.1**	*b*	*a*	*a*	*a*	*a*	*a*
	100	*a*	*b*	*c*	*c*		*c*
	0.1 (Pre-treated)*	*b*	*b*	*b*	*b*	*b*	*b*

Table 8. Statistical analysis performed for pH variation. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)					
		0	**90**	**210**	**360**	**510**	**600**
Pressure (MPa)	**0.1**	*b*	*c*	*c*	*c*	*b*	*c*
	100	*b*	*a*	*a*	*a*		*a*
	0.1 (Pre-treated)*	*a*	*b*	*b*	*b*	*a*	*b*

Table 9. Statistical analysis performed for reducing sugars concentration. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)					
		0	**90**	**210**	**360**	**510**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*b*	*a*	*c*
	100	*a*	*a*	*a*	*a*		*a*
	0.1 (Pre-treated)*	*a*	*a*	*a*	*ab*	*a*	*b*

c) Monitoring Specific Physicochemical Parameters during Yogurt's Production

i. D-Glucose Concentration

Table 10. Statistical analysis performed for D-glucose concentration. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*b*	*c*	*b*	*d*
	5	*b*	*a*	*b*	*c*
	100	*b*	*b*	*a*	*a*
	0.1 (Pre-treated)*	*a*			*b*

ii. L-/D-Lactic Acid Concentration

Table 11. Statistical analysis performed for L-lactic acid concentration. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*b*	*a*	*a*	*a*
	5	*b*	*b*	*b*	*a*
	100	*b*	*c*	*c*	*b*
	0.1 (Pre-treated)*	*a*			*a*

Table 12. Statistical analysis performed for D-lactic acid concentration. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**		*a*	*a*	*a*
	5		*a*	*a*	*a*
	100				
	0.1 (Pre-treated)*				

iii. Acetaldehyde Concentration

Table 13. Statistical analysis performed for acetaldehyde concentration. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*a*
	5	*a*	*a*	*b*	*a*
	100	*a*	*b*	*ab*	*a*
	0.1 (Pre-treated)*	*a*			*a*

d) Microbiological Analysis of Yogurt's Fermentative Bacteria

Table 14. Statistical analysis performed for *Streptococcus thermophilus* count. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*a*
	5	*a*	*a*	*a*	*b*
	100	*a*	*b*		*d*
	0.1 (Pre-treated)*	*b*			*c*

Table 15. Statistical analysis performed for *Lactobacillus bulgaricus* count. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*a*
	5	*a*	*a*	*b*	*b*
	100	*a*			
	0.1 (Pre-treated)*				*ab*

Appendix III:

Activation Volume Calculations

a) Titratable Acidity

To perform activation volumes calculation, several values of titratable acidity variation along fermentation time (at different pressure conditions) with linear behavior were selected and its napierian logarithm was calculated (Figure 1).

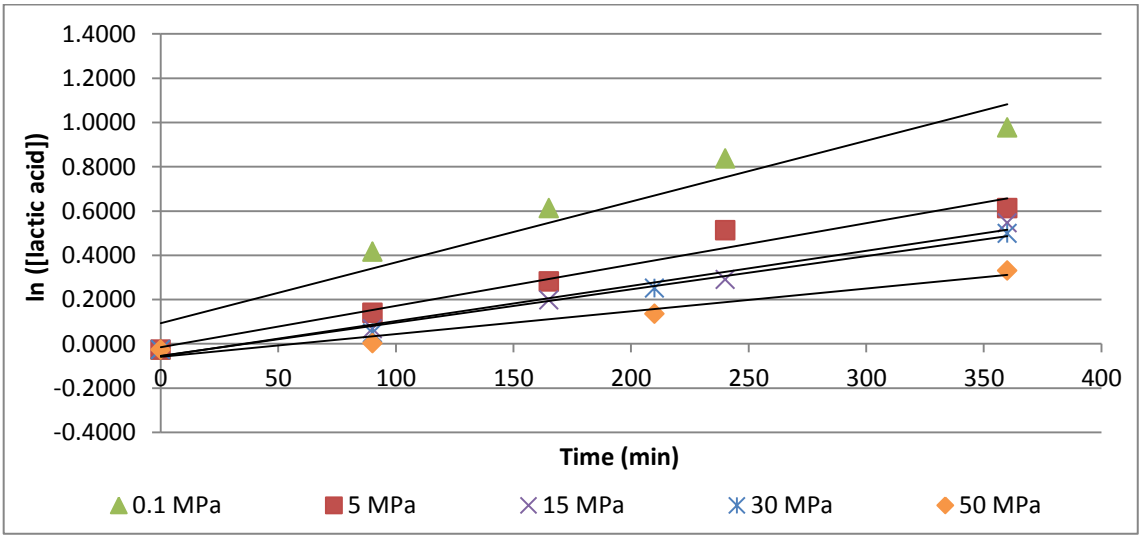

Figure 1. Napierian logarithm of lactic acid concentration during fermentation time, under different pressure conditions.

Table 1 was constructed using the slopes of each series shown at Figure 1 as the reaction constant rate (k) and assuming that R_p = 8.314 (cm^3.MPa)/(K.mol) and T = 316.15 K.

Table 1. Rate constant of titratable acidity and its napierian logarithm calculation, for different pressure conditions.

Pressure (MPa)	Pressure/(R_p*T)	ln(Titratable Acidity) vs. Time		
		|m| = k	r^2	ln(k)
5	1.9E-03	0.00187	0.968	-6.282
15	5.7E-03	0.00159	0.982	-6.446
30	1.1E-02	0.00150	0.988	-6.503
50	1.9E-02	0.00103	0.964	-6.881

The values shown in Table 1 were then used to calculate the linear relation present in Figure 2, which slope corresponds to the activation volume value obtained to titratable acidity (32.98 cm^3/mol).

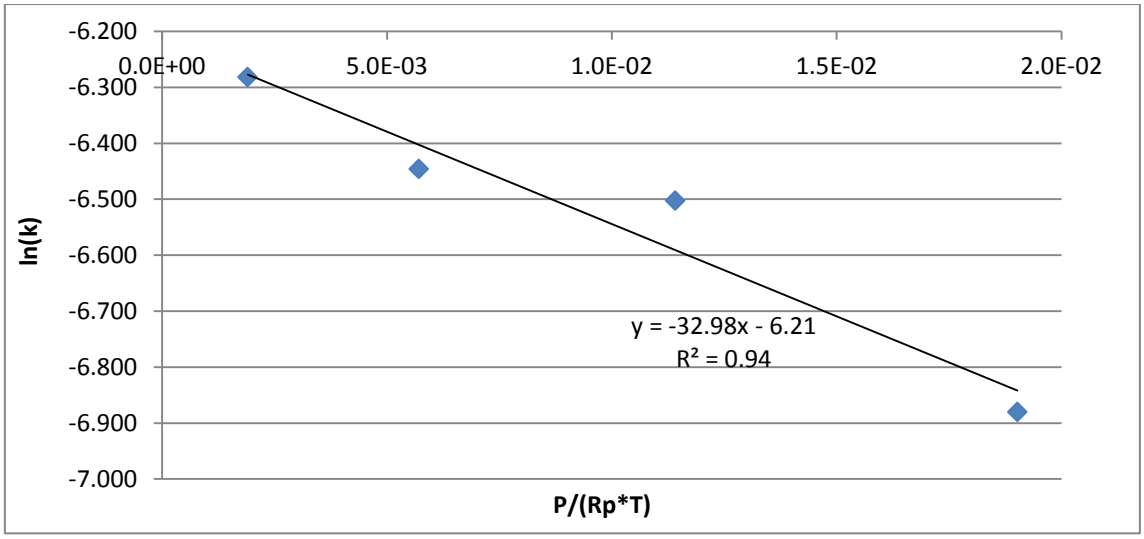

Figure 2. Volume activation (Va) calculation for lactic acid concentration during fermentation time, which corresponds to the slope of linear regression.

b) H^+ concentration

To perform activation volumes calculation, several values of pH variation along fermentation time (at different pressure conditions) with linear behavior were selected.

Using the pH values it was possible to calculate the concentration of H^+ and its respective napierian logarithm, represented in Figure 3.

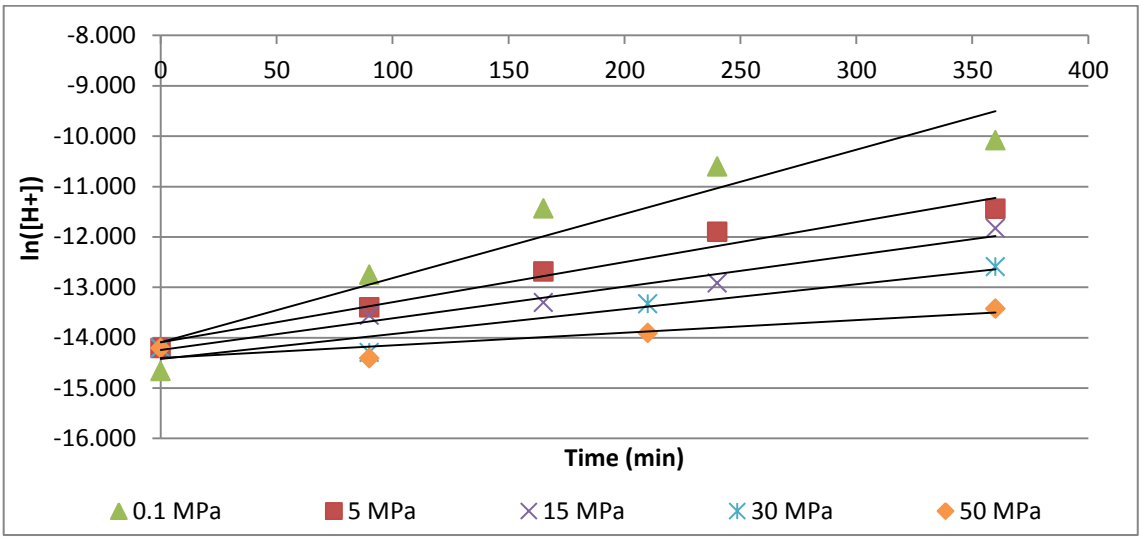

Figure 3. Napierian logarithm of H^+ concentration during fermentation time, under different pressure conditions.

Table 2 was constructed using the slopes of each series shown at Figure 3 as the reaction constant rate (k) and assuming that R_p = 8.314 (cm^3.MPa)/(K.mol) and T = 316.15 K.

Table 2. Rate constant of H^+ concentration and its napierian logarithm calculation, for different pressure conditions.

Pressure (MPa)	Pressure/(R_p*T)	ln([H^+]) vs. Time		
		|m| = k	r^2	ln(k)
5	1.9E-03	7.95E-03	0.971	-4.835
15	5.7E-03	6.28E-03	0.974	-5.070
30	1.1E-02	4.95E-03	0.920	-5.309
50	1.9E-02	2.49E-03	0.817	-5.995

The values shown in Table 2 were then used to calculate the linear relation present in Figure 4, which slope corresponds to the activation volume value obtained to pH (66.33 cm^3/mol).

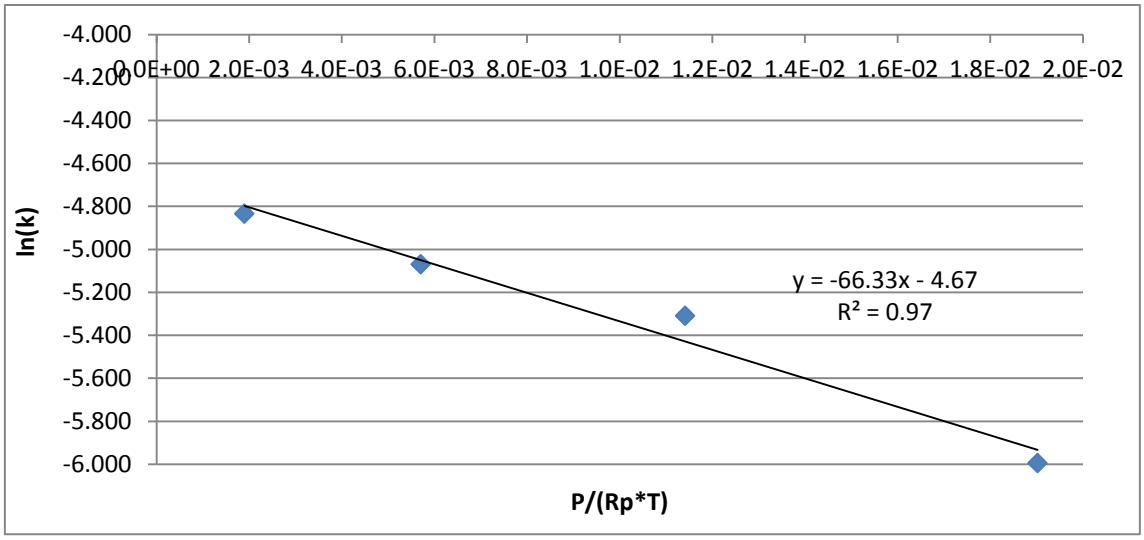

Figure 4. Volume activation (Va) calculation for H^+ concentration during fermentation time, which corresponds to the slope of linear relation.

c) Reducing Sugars Concentration

To perform activation volumes calculation, several values of reducing sugars concentration along fermentation time (at different pressure conditions) with linear behavior were selected and its napierian logarithm was calculated (Figure 5).

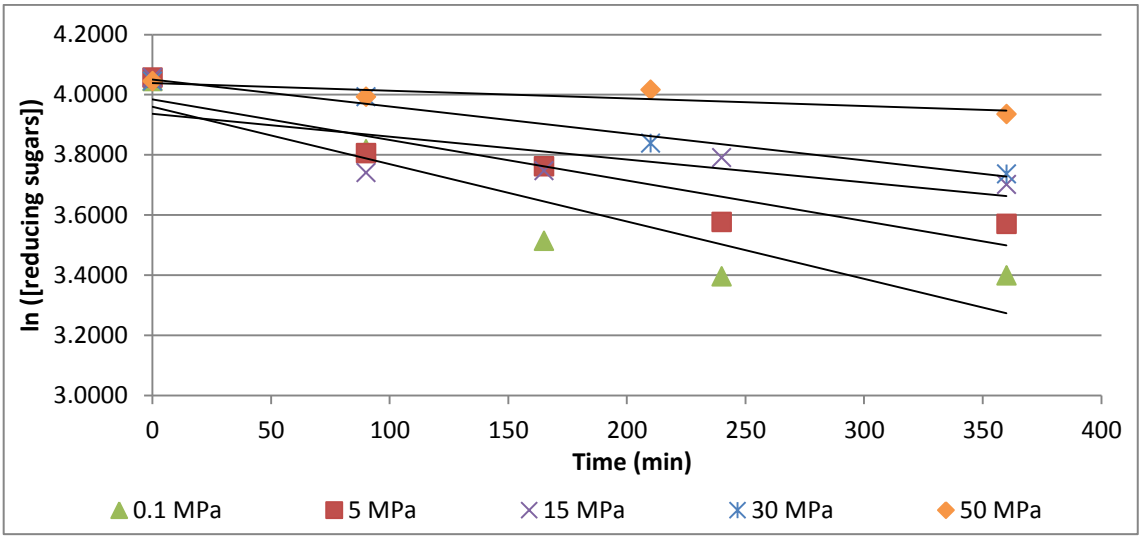

Figure 5. Napierian logarithm of reducing sugars concentration during fermentation time, under different pressure conditions.

Table 3 was constructed using the slopes of each series shown at Figure 5 as the reaction constant rate (*k*) and assuming that R_p = 8.314 (cm^3.MPa)/(K.mol) and T = 316.15 K.

Table 3. Rate constant of reducing sugars concentration and its napierian logarithm calculation, for different pressure conditions.

Pressure (MPa)	Pressure/(R_p*T)	ln(Reducing Sugars) vs. Time		
		|m| = k	r^2	ln(k)
5	1.9E-03	0.00135	0.870	-6.608
15	5.7E-03	0.00076	0.540	-7.180
30	1.1E-02	0.00090	0.981	-7.015
50	1.9E-02	0.00026	0.741	-8.269

The values shown in Table 3 were then used to calculate the linear relation present in Figure 6, which slope corresponds to the activation volume value obtained to reducing sugars concentration (86.47 cm^3/mol).

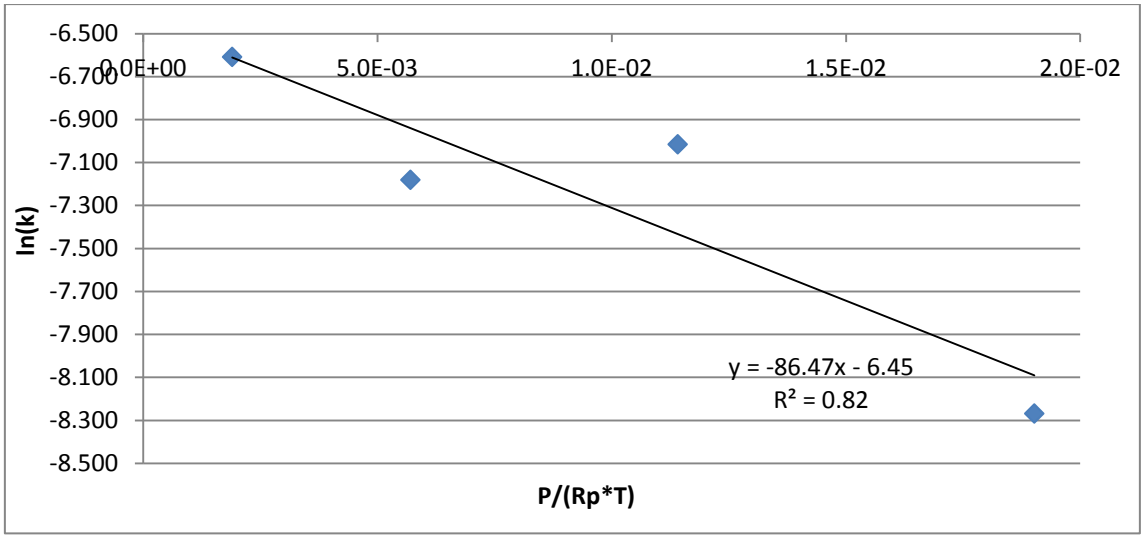

Figure 6. Volume activation (Va) calculation for reducing sugars concentration during fermentation time, which corresponds to the slope of linear relation.

Appendix IV:

Estimation of Maximal Glucose Concentration in Yogurt

According to literature [1], the percentage of lactose in milk is ≈ 5% (in weight) and semi-skimmed milk density at 20 ^{o}C is 1.020 Kg/L. So, according to these values, there are 5 mg of lactose in 0.098 mL of milk.

In the beginning of fermentation, each sample has 7.5 mL in volume which equals to 0.38 g of lactose (corresponding to 1.1×10^{-3} mol), which can be consumed by lactic acid bacteria. Assuming that all lactose present in milk is hydrolyzed by reaction represented at Figure 1, in the end there is 1.1×10^{-3} mol of glucose, which corresponds to 0.20 g, since in reaction there is a proportion of 1 mol of lactose to 1 mol of glucose.

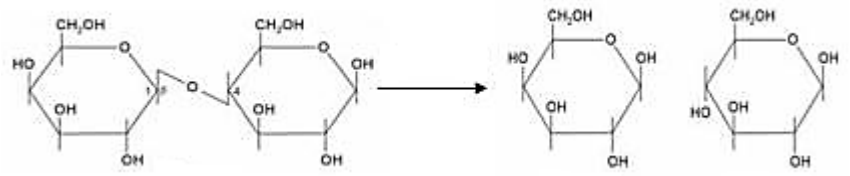

Figura 1. Lactose hydrolysis.

The D-glucose amount present in sample with the maximum D-glucose concentration obtained in this work (600 minutes at 100 MPa, 1.54 g/L of supernatant) was calculated and it was concluded that in this sample was present 0.011 g of D-glucose (practical value). Comparing this value with the D-glucose amount obtained if lactose were total hydrolyzed (0.20 g), it is possible to verify that the practical value is only ≈ 5 % of this value.

Appendix V:

Determination of L-:D-Lactic Acid Ratios

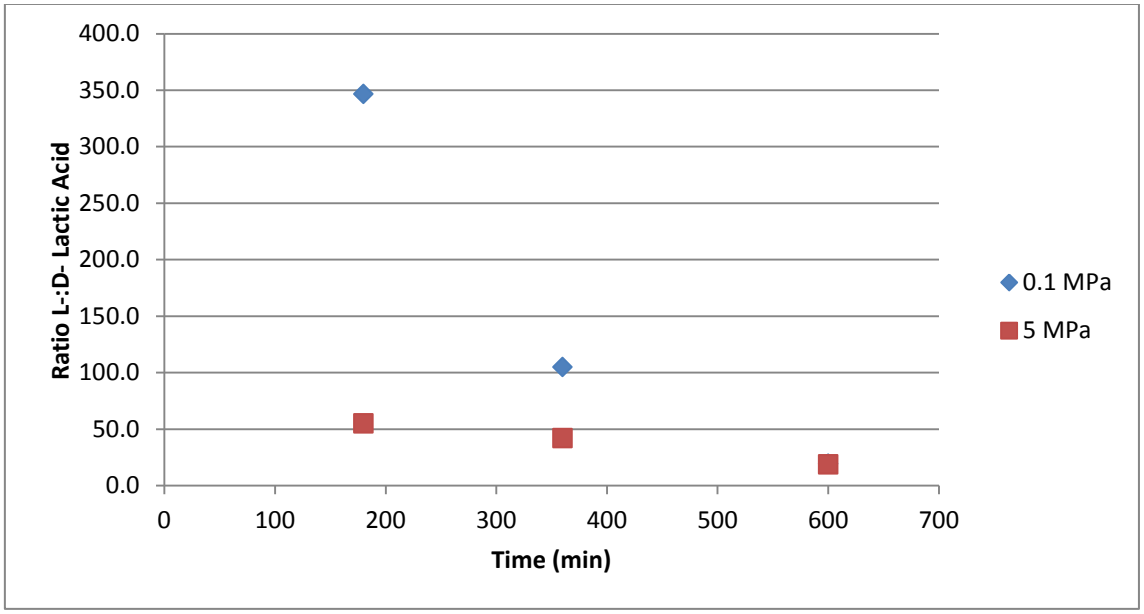

Figure 1. Ratios of L-:D-lactic acid concentrations during fermentation time.

Note: The samples for which the correspondent D-lactic acid presence was not detected are not represented in Figure 1, because it's impossible to calculate the L-:D-lactic acid ratio.